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(54) Title: NOVEL PLANT STEROID 5α REDUCTASE, DET2

(57) Abstract

A novel plant steroid 5α reductase, DET2, is provided, as well as polynucleotides encoding DET2. DET2 or mammalian steroid 5α-reductase is useful in promoting increased plant yield and/or increased plant biomass. Genetically modified plants charaterized as having increased yield and methods for producing such plants are also provided.

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NOVEL PLANT STEROID 5α REDUCTASE, DET2

Field of the Invention

The present invention relates generally to plant genetic engineering, and specifically to producing genetically engineered plants characterized as having a phenotype of increased crop yield.

Background of the Invention

Plant growth and development are governed by complex interactions between environmental signals and internal factors. Light regulates many developmental processes throughout the plant life cycle, from seed germination to floral induction (Chory, J. *Trends Genet.*, 9:167, 1993; McNellis and Deng, *Plant Cell*, 7:1749, 1995), and causes profound morphological changes in young seedlings. In the presence of light, hypocotyl growth is inhibited, cotyledons expand, leaves develop, chloroplasts differentiate, chlorophylls are produced, and many light-inducible genes are coordinately expressed. It has been suggested that plant hormones, which are known to affect the division, elongation, and differentiation of cells, are directly involved in the response of plants to light signals (P.J. Davies, *Plant Hormones: Physiology, Biochemistry and Molecular Biology*, pp 1-836 , 1995; Greef and Freddericq, Photomorphogenesis, pp 401-427, 1983). The interactions between phototransduction pathways and plant hormones however are not well understood.

The brassinosteroids are a unique class of biologically active natural products that possess high specific activity and plant steroidal hormone activity. Their low effective concentrations for use on crops make them environmentally safe and those brassinosteroids used on a large scale are generally non-toxic. At the physiological level, brassinosteroids elicit many changes and could represent a new class of hormones in plants. The economic aspects of the brassinosteroids may have worldwide effects. The

brassinosteroids can be used as plant protectants from both pesticide and environmental adversity. In addition, brassinosteroids appear to be important for insect control. Further, brassinosteroids may regulate some stage of the reproductive cycle in plants, and other species, thereby providing the means to increase or decrease the reproductive process. For example, in certain horticultural crops, it may be desirable to eliminate the flowering process to ensure continuous production of other tissues such as leaves, bulbs and other storage organs. This modulation of the reproductive process could be important in the control of certain seed bearing weeds, where cessation of the flowering cycle eliminates proceeding generations. Brassinosteroids also appear to stimulate root growth, and external application causes no deformity of plants.

Brassinosteroids qualify for classification as biochemical pesticides. Such pesticides are generally distinguished from conventional chemical pesticides by their unique modes of action, low effective concentration, target species, and specificity. Historically, the brassinosteroids have not been used in actual agricultural applications due to the expense involved in producing them as well as the difficulty in purifying them.

Summary of the Invention

Although steroid hormones are important for animal development, the physiological role of plant steroids is largely unknown. The present invention is based on the discovery of the DET2 gene, which encodes a protein that shares significant sequence identity with mammalian steroid 5α-reductases and is involved in the brassinolide biosynthetic pathway. A mutation of glutamate 204, which is required for the activity of human steroid reductase, abolishes the *in vivo* activity of DET2 and leads to defects in light-regulated development. These defects can be ameliorated by application of the plant steroid, brassinolide.

In a first embodiment, the invention provides DET2 polypeptide and isolated polynucleotide sequences encoding DET2.

In another embodiment, the invention provides a method for producing a genetically modified plant characterized as having increased yield as compared to a wild-type plant. The method includes transferring at least one copy of a DET2-encoding polynucleotide or a polynucleotide encoding another steroid 5α reductase (e.g., mammalian) operably associated with a promoter to a plant cell to obtain a transformed plant cell and producing a plant from the transformed plant cell. Such genetically modified plants may exhibit increased crop yield or increased biomass, for example.

In yet another embodiment of the invention provides a method for producing a plant characterized as having increased yield by contacting a plant with having a native DET2 gene operably linked to its native promoter, with a promoter-inducing amount of an agent which induces DET2 gene expression, wherein induction of DET2 gene expression results in production of a plant having increased yield as compared to a plant not contacted with the inducing agent. Thus, transcription factors or chemical agents may be used to increase expression of DET2 in a plant, in order to provide increased yield.

Brief Description of the Drawings

Figure 1 is a schematic illustration of the cloning and sequence analysis of the *DET2* gene. Figure 1A shows a summary of positional cloning. Three classes of cDNA were identified from an *Arabidopsis* cDNA library with cosmid 217-61 as a probe, and their relative positions and transcriptional directions (5'-3') are indicated. Figure 1B shows a map of the gene structure of *DET2* and mutations in the *DET2* gene. Thick lines indicate exons, and the open box denoted an intron. Positions of mutations are relative to the initiation codon. Z, stop codon. Figure 1C shows the nucleotide and deduced amino acid sequence of DET2 (SEQ ID NO:1 and 2, respectively).

Figure 2 shows a photo of light-grown 12-day-old seedlings (Figure 2A) and dark-grown 10-day-old seedlings (Figure 2B) after complementation of *det2* by the wild-type *DET2* gene. (From left to right in each panel) Wild-type Col-0, *det2*-1, and transgenic *det2*-1 containing cosmid 217-61.

Figure 3 shows a sequence comparison of *DET2* with mammalian steroid 5α-reductases. Figure 3A is the deduced amino acid sequence of the *DET2* gene aligned with the steroid 5α-reductases from rat (rS5R1 and rS5R2) and human (hS5R1 and hS5R2). Dashes indicate gaps introduced to maximize alignment, and residues conserved in at least two of the five sequences are shaded. The arrow indicates the glutamate mutated in *det2*-1 and *det2*-6 alleles. (Single-letter abbreviation for the amino acid residues are as follows: A, Alta; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gin; R, Arg; S, Ser; T, The; V, Val; W, Trp; and Y, Tyr). Figure 3B shows a phylogenetic analysis of the relationship between *DET2* protein and mammalian steroid 5α-reductases. The scale measures the relative distance between sequences.

Figure 4A shows the proposed function of *DET2* protein in the brassinolide biosynthetic pathway. Asterisk (*) indicates six intermediate steps (Fujicka, *et al., supra*).

Figure 4B shows dark-grown 10-day-old Arabadopsis seedlings and Figure 4C shows light-grown 12-day-old seedlings. Shown from left to right in each panel are wild-type, det2-1, and brassinolide-treated *det*2-1 plants.

Figure 4D shows a dose-response of brassinolide-induced hypocotyl elongation of dark-grown seedlings and light-grown wild-type plants. Data represent the mean ± SE obtained from triplicate determinations, each with an average sample size of 12 seedlings.

Figure 5 shows chemical reactions catalyzed by mammalian steroid 5α reductases (panel A) and *Arabidopsis* DET2 (panel B).

Figure 6 shows *Arabidopsis* DET2 has steroid 5α reductase activity. Thin layer chromatography analysis of steroids produced by incubating 64 nM [^{14}C]progesterone with human embryonic 293 cells transfected with a pCMV5 expression plasmid containing cDNA of human type 1 steroid 5α-reductase (lane 1), no insert (lane 2), wild-type *DET2* (lane 3), or *det2-1* (lane 4) is shown.

Figure 7 shows biochemical characterization of the steroid 5α-reductase activity of DET2.

Figure 7A is a Lineweaver-Burk plot for [^{14}C]testosterone.

Figure 7B is a Lineweaver-Burk plot for [^{14}C]progesterone.

Figure 7C shows competitive inhibition of DET2 enzyme activity by 4-MA. Steroid 5α-reductase activity was assayed in the presence of the indicated concentration of 4-MA and either 0.34 *m*M or 0.68 *m*M [^{14}C]progesterone. The intersection of the two lines defines the K_i (Dixon, M. & Webb, E.C., (1979) *Enzymes* Academic, New York).

Figure 7D shows a pH profile of the DET2 steroid 5α-reductase activity using [^{14}C]testosterone as a substrate. Assays were carried out at the indicated pHs in the presence of 5 *m*g of cell lysate protein, 1 *m*M [^{14}C]testosterone, and 2.0 mM NADPH for 20 min at 37°C.

Figure 8 shows human steroid 5α-reductase can complement the *det2-1* mutation.

Figure 8A shows schematic representations of pMD-hS5R expression plasmids used to transform *det2-1* mutants. Two constructs were made for each type of human steroid 5α-reductase cDNA: a shorter one containing a truncated 3'-untranslated region and a longer one containing a full length human cDNA. DNA fragments indicated are human steroid 5α-reductase (hS5R), nopaline synthase promoter (NosPro) and transcriptional terminator (Nos-ter), neomycin phosphotransferase gene (NPTII), cauliflower mosaic virus 35S promoter (35Spro), and multiple cloning sites (MCS).

Figure 8B-D shows complementation of *det2-1* mutation by human steroid 5α-reductase cDNA.

Figure 8B Dark-grown 7-day-old seedlings.

Figure 8C Light-grown 7-day-old seedlings.

Figure 8D Light-grown 3-week-old seedlings. (From left to right in panel B, C, and D) Wild-type Col-0, *det2-1*, transgenic *det2-1* containing the full-length human steroid 5α-reductase type 1 cDNA and transgenic *det2-1* containing the full-length human steroid 5α-reductase type 2 cDNA.

Figure 9 shows the effect of 4-MA on the hypocotyl lengths of *det2-1*, wild-type and transgenic *det2-1* plants. Data represent the mean ± SE obtained from 25 seedlings of each genotype.

Figure 10 shows the expression level of human steroid 5α-reductase cDNA is correlated with phenotype in transgenic *det2-1* plants.

Figure 10A shows hypocotyl length of dark-grown seedlings of wild-type Col-0, *det2-1* and segregating progeny of primary transgenic *det2-1* plants. Data represent the mean ±SE obtained from a population with an average sample size of 60 seedlings.

Figure 10B shows a Northern blot analysis of transgene expression. Each lane contained 20 mg of total plant RNA.

Figure 10C shows the Morphology of 14-day-old light-grown seedlings. (From left to right in each panel), 1, *det2-1*; 2, hS5R1-1.3kb(04); 3, hS5R1-1.3kb(08); 4,

hS5R1-2.1kb(04); 5, hS5R1-2.1kb(17); 6, wild-type; 7, hS5R2-0.8kb(01); 8, hS5R2-0.8kb(12); 9, hS5R2-0.8kb(15); 10, hS5R2-2.4kb(04); 11, hS5R2-2.4kb(05); 12, hS5R2-2.4kb(12).

Description of the Preferred Embodiments

The present invention provides a novel steroid 5α-reductase, DET2, which is involved in the synthesis of the plant steroid hormone, brassinolide. Overexpression of DET2 reductase or mammalian steroid 5α-reductase (e.g., human, monkey, rat, mouse) in transgenic plants causes such plants to become significantly larger and more robust than their wild-type counterparts, thus increasing plant yields.

As used herein, the term "yield" or "plant yield" refers to increased plant growth, increased crop growth, and/or increased biomass production.

In a first embodiment, the present invention provides a substantially pure DET2 polypeptide. DET2 polypeptide is exemplified by the amino acid sequence shown in FIGURE 1C and SEQ ID NO:2. DET2 polypeptide is characterized as having a predicted molecular weight of 31 kDa as determined by SDS-PAGE, having steroid 5α reductase activity and functioning in the brassinolide biosynthetic pathway.

The deduced amino acid sequence of the *DET2* gene is similar to that of mammalian steroid 5α-reductases, with 38 to 42% sequence identity. The sequence similarity increases to 54 to 60% when conservative substitutions are taken into account. Two isozymes (types 1 and 2) of steroid 5α-reductase have been isolated in rats and humans (Wilson, *et al., Endocr. Rev.*, 14:577, 1993; Russell and Wilson, *Annu. Rev. Biochem.*, 63:25, 1994). Phylogenetic analysis shows that *DET2* is at least as closely related to type 2 enzymes as type 2 enzymes are related to type 1 enzymes.

The term "substantially pure" as used herein refers to DET2 polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify DET2 using standard techniques for protein purification. The substantially pure polypeptide will yield a single major band of about 31kD on a denaturing polyacrylamide gel. The purity of the DET2 polypeptide can also be determined by amino-terminal amino acid sequence analysis.

The invention includes functional DET2 polypeptide, and functional fragments thereof. As used herein, the term "functional polypeptide" refers to a polypeptide which

possesses biological function or activity which is identified through a defined functional assay and which is associated with a particular biologic, morphologic, or phenotypic alteration in the cell. The term "functional fragments of DET2 polypeptide", refers to all fragments of DET2 that retain DET2 activity, *e.g.*,steroid 5α-reductase activity. Biologically functional fragments, for example, can vary in size from a polypeptide fragment as small as an epitope capable of binding an antibody molecule to a large polypeptide capable of participating in the characteristic induction or programming of phenotypic changes within a cell.

The steroid 5α-reductase activity of DET2 and the role of in the brassinolide biosynthetic pathway can be utilized in bioassays to identify biologically active fragments of DET2 polypeptide or related polypeptides. For example, DET2 may catalyze the conversion of campesterol to campestanol, therefore an assay can be performed to detect DET2 enzymatic activity. Inhibitors of DET2 could be used to cause loss of function of DET2 resulting in, for example, male sterile plants, reduced stature, etc. For example, inhibition of DET2 is useful in horticulture for creating dwarf varieties.

Minor modifications of the DET2 primary amino acid sequence may result in proteins which have substantially equivalent activity to the DET2 polypeptide described herein in SEQ ID NO:2 (Figure 1C). Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of DET2 is present, *e.g.*, steroid 5α reductase activity is present to promote increased plant or crop yield and/or biomass. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its activity. This can lead to the development of a smaller active molecule which could have broader utility. For example, it may be possible to remove amino or carboxy terminal amino acids required for DET2 activity.

DET2 polypeptide includes amino acid sequences substantially the same as the sequence set forth in SEQ ID NO:2. The term "substantially the same" refers to amino

acid sequences that retain the activity of DET2 as described herein, *e.g.*, steroid 5α reductase activity. The DET2 polypeptides of the invention include conservative variations of the polypeptide sequence. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Invention proteins can be analyzed by standard SDS-PAGE and/or immuno-precipitation analysis and/or Western blot analysis, for example. In addition, the *in vitro* synthesized (IVS) protein assay as described in the present examples can be used to analyze DET2 protein product.

The invention also provides an isolated polynucleotide sequence encoding a polypeptide having the amino acid sequence of SEQ ID NO:2. The DET2 gene has been mapped to a 150-kb interval on *Arabidopsis* chromosome 2. The *DET2* transcript contains a single, long open reading frame that encodes a 262-amino acid protein. The term "isolated" as used herein includes polynucleotides substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which it is naturally associated. Polynucleotide sequences of the invention include DNA, cDNA and RNA sequences which encode DET2. It is understood that polynucleotides encoding all or varying portions of DET2 are included herein, as long as they encode a polypeptide with DET2 activity. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides as well as splice variants. For example, portions of the mRNA sequence may be altered due to alternate RNA splicing patterns or the use of alternate promoters for RNA transcription. Moreover, DET2 polynucleotides

of the invention include polynucleotides having alterations in the nucleic acid sequence which still encode functional DET2. Alterations in DET2 nucleic acid include but are not limited to intragenic mutations (*e.g.*, point mutation, nonsense (stop), antisense, splice site and frameshift) and heterozygous or homozygous deletions. Detection of such alterations can be done by standard methods known to those of skill in the art including sequence analysis, Southern blot analysis, PCR based analyses (*e.g.*, multiplex PCR, sequence tagged sites (STSs)) and *in situ* hybridization. Invention polynucleotide sequences also include antisense sequences. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of DET2 polypeptide encoded by such nucleotide sequences retains DET2 steroid 5α reductase activity. A "functional polynucleotide" denotes a polynucleotide which encodes a functional polypeptide as described herein. In addition, the invention also includes a polynucleotide encoding a polypeptide having the biological activity of an amino acid sequence of SEQ ID NO:2 and having at least one epitope for an antibody immunoreactive with DET2 polypeptide.

As used herein, the terms polynucleotides and nucleic acid sequences of the invention refer to DNA, RNA and cDNA sequences.

The polynucleotide encoding DET2 includes the nucleotide sequence in FIGURE 1C (SEQ ID NO:1), as well as nucleic acid sequences complementary to that sequence. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxyribonucleotides A, G, C, and T of FIGURE 1C are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments ("probes") of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the probe to selectively hybridize to DNA that encodes the protein of FIGURE 1C (SEQ ID NO: 2). "Selective hybridization" as used herein refers to hybridization under moderately stringent or highly stringent

physiological conditions (See, for example, the techniques described in Maniatis et al., 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference), which distinguishes related from unrelated DET2 nucleotide sequences.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

Specifically disclosed herein is a cDNA sequence for DET2. Figure 1C shows the complete cDNA and deduced protein sequences (SEQ ID NO:1 and 2, respectively).

DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization or computer-based techniques which are well known in the art. Such techniques include, but are not limited to: 1) hybridization of genomic or cDNA libraries with probes to detect homologous nucleotide sequences; 2) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features; 3) polymerase chain reaction (PCR) on genomic DNA or cDNA using

primers capable of annealing to the DNA sequence of interest; 4) computer searches of sequence databases for similar sequences; and 5) differential screening of a subtracted DNA library.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the DET2 sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of the amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucl. Acid Res.*, 9:879, 1981). Alternatively, a subtractive library, as illustrated herein is useful for elimination of non-specific cDNA clones.

Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present

in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al., Nucl. Acid Res.*, 11:2325, 1983).

A cDNA expression library, such as lambda gt11, can be screened indirectly for DET2 peptides using antibodies specific for DET2. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of DET2 cDNA.

DNA sequences encoding DET2 or other 5α-reductases can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny or graft material, for example, of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the DET2 polynucleotide or other (e.g., mammalian steroid 5α-reductase) sequences may be inserted into a recombinant expression vector. The terms "recombinant expression vector" or "expression vector" refer to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the DET2 genetic sequence. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted DET2 sequence. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the DET2 coding sequence or for example mammalian steroid 5α-reductase coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques.

A variety of host-expression vector systems may be utilized to express the DET2 coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the DET2 coding sequence; yeast transformed with recombinant yeast expression vectors containing the DET2 coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the DET2 coding sequence; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the DET2 coding sequence; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, retroviruses, adenovirus, vaccinia virus) containing the DET2 coding sequence, or transformed animal cell systems engineered for stable expression.

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see *e.g.*, Bitter et al., 1987, Methods in Enzymology 153:516-544). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage γ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted DET2 coding sequence.

Isolation and purification of recombinantly expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

The invention also includes antibodies immunoreactive with DET2 polypeptide or antigenic fragments thereof. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in the art (Kohler, *et al.*, *Nature*, 256:495, 1975).

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')2, and Fv which are capable of binding to an epitopic determinant present in DET2 polypeptide. Such antibody fragments retain some ability to selectively bind with its antigen or receptor.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988), incorporated herein by reference).

As used in this invention, the term "epitope" refers to an antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Antibodies which bind to the DET2 polypeptide of the invention can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. For example, it may be desirable to produce antibodies that specifically bind to the N- or C-terminal domains of DET2. The polypeptide or peptide used to immunize an animal which is derived from translated cDNA or chemically synthesized which can be conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the immunizing peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid.

Invention polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See for example, Coligan, *et al.*, Unit 9, Current Protocols in Immunology, Wiley Interscience, 1994, incorporated by reference).

It is also possible to use the anti-idiotype technology to produce invention monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

In another embodiment, the invention provides a method for producing a genetically modified plant characterized as having increased yield as compared to a plant which has not been genetically modified (*e.g.*, a wild-type plant). The term "yield" has been previously defined herein. The method includes the steps of contacting a plant cell with at least one vector containing at least one nucleic acid sequence encoding a steroid 5α-reductase, e.g., DET2 or a mammalian enzyme (*e.g.*, human type 1 or type 2 steroid 5α-reductase), wherein the nucleic acid sequence is operably associated with a promoter, to obtain a transformed plant cell; producing a plant from the transformed plant cell; and thereafter selecting a plant exhibiting increased yield.

The term "genetic modification" as used herein refers to the introduction of one or more heterologous nucleic acid sequences, *e.g.*, DET2 encoding sequence or human type 1 or type 2 5α-reductase, into one or more plant cells, which can generate whole, sexually competent, viable plants. The term "genetically modified" as used herein refers to a plant which has been generated through the aforementioned process. Genetically modified plants of the invention are capable of self-pollinating or cross-pollinating with other plants of the same species so that the foreign gene, carried in the germ line, can be

inserted into or bred into agriculturally useful plant varieties. The term "plant cell" as used herein refers to protoplasts, gamete producing cells, and cells which regenerate into whole plants. Accordingly, a seed comprising multiple plant cells capable of regenerating into a whole plant, is included in the definition of "plant cell".

As used herein, the term "plant" refers to either a whole plant, a plant part, a plant cell, or a group of plant cells, such as plant tissue, for example. Plantlets are also included within the meaning of "plant". Plants included in the invention are any plants amenable to transformation techniques, including angiosperms, gymnosperms, monocotyledons and dicotyledons.

Examples of monocotyledonous plants include, but are not limited to, asparagus, field and sweet corn, barley, wheat, rice, sorghum, onion, pearl millet, rye and oats. Examples of dicotyledonous plants include, but are not limited to tomato, tobacco, cotton, rapeseed, field beans, soybeans, peppers, lettuce, peas, alfalfa, clover, cole crops or *Brassica oleracea* (e.g., cabbage, broccoli, cauliflower, brussel sprouts), radish, carrot, beets, eggplant, spinach, cucumber, squash, melons, cantaloupe, sunflowers and various ornamentals. Woody species include poplar, pine, sequoia, cedar, oak, etc.

The term "heterologous nucleic acid sequence" as used herein refers to a nucleic acid foreign to the recipient plant host or, native to the host if the native nucleic acid is substantially modified from its original form. For example, the term includes a nucleic acid originating in the host species, where such sequence is operably linked to a promoter that differs from the natural or wild-type promoter. In the broad method of the invention, at least one nucleic acid sequence encoding DET2 or other steroid 5α reductase (e.g., mammalian enzyme) is operably linked with a promoter. It may be desirable to introduce more than one copy of DET2 or other steroid 5α-reductase polynucleotide into a plant for enhanced expression of the corresponding polypeptide. For example, multiple copies of the DET2 gene would have the effect of increasing production of DET2 in the plant. It should be understood that the steroid 5α-reductase encoding nucleic acid sequence refers

to DET2, human type 1 or type 2 5α-reductase, and other steroid 5α-reductases. The method of the invention also includes any combination of these enzymes.

Genetically modified plants of the present invention are produced by contacting a plant cell with a vector including at least one nucleic acid sequence encoding DET2. To be effective once introduced into plant cells, the DET2 nucleic acid sequence must be operably associated with a promoter which is effective in the plant cells to cause transcription of DET2. Additionally, a polyadenylation sequence or transcription control sequence, also recognized in plant cells may also be employed. It is preferred that the vector harboring the nucleic acid sequence to be inserted also contain one or more selectable marker genes so that the transformed cells can be selected from non-transformed cells in culture, as described herein.

The term "operably associated" refers to functional linkage between a promoter sequence and the DET2 nucleic acid sequence regulated by the promoter. The operably linked promoter controls the expression of the DET2 nucleic acid sequence.

The expression of structural genes employed in the present invention may be driven by a number of promoters. Although the endogenous, or native promoter of a structural gene of interest may be utilized for transcriptional regulation of the gene, preferably, the promoter is a foreign regulatory sequence. For plant expression vectors, suitable viral promoters include the 35S RNA and 19S RNA promoters of CaMV (Brisson, *et al.*, *Nature*, 310:511, 1984; Odell, *et al.*, *Nature*, 313:810, 1985); the full-length transcript promoter from Figwort Mosaic Virs (FMV) (Gowda, *et al.*, *J. Cell Biochem.*, 13D: 301, 1989) and the coat protein promoter to TMV (Takamatsu, *et al.*, *EMBO J.* 6:307, 1987). Alternatively, plant promoters such as the light-inducible promoter from the small subunit of ribulose bis-phosphate carboxylase (ssRUBISCO) (Coruzzi, *et al.*, *EMBO J.*, 3:1671, 1984; Broglie, *et al.*, *Science*, 224:838, 1984); mannopine synthase promoter (Velten, *et al.*, *EMBO J.*, 3:2723, 1984) nopaline synthase (NOS) and octopine synthase (OCS) promoters (carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*) or

heat shock promoters, *e.g.*, soybean hsp17.5-E or hsp17.3-B (Gurley, *et al., Mol. Cell. Biol.*, 6:559, 1986; Severin, *et al., Plant Mol. Biol.*, 15:827, 1990) may be used.

Promoters useful in the invention include both natural constitutive and inducible promoters as well as engineered promoters. The CaMV promoters are examples of constitutive promoters. To be most useful, an inducible promoter should 1) provide low expression in the absence of the inducer; 2) provide high expression in the presence of the inducer; 3) use an induction scheme that does not interfere with the normal physiology of the plant; and 4) have no effect on the expression of other genes. Examples of inducible promoters useful in plants include those induced by chemical means, such as the yeast metallothionein promoter which is activated by copper ions (Mett, *et al., Proc. Natl. Acad. Sci., U.S.A.*, 90:4567, 1993); In2-1 and In2-2 regulator sequences which are activated by substituted benzenesulfonamides, *e.g.*, herbicide safeners (Hershey, *et al., Plant Mol. Biol.*, 17:679, 1991); and the GRE regulatory sequences which are induced by glucocorticoids (Schena, *et al., Proc. Natl. Acad. Sci., U.S.A.*, 88:10421, 1991). Other promoters, both constitutive and inducible will be known to those of skill in the art.

The particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of structural gene product, *e.g.*, DET2, to cause increased yield and/or increased biomass. The promoters used in the vector constructs of the present invention may be modified, if desired, to affect their control characteristics.

Tissue specific promoters may also be utilized in the present invention. An example of a tissue specific promoter is the promoter active in shoot meristems (Atanassova, *et al., Plant J.*, 2:291, 1992). Other tissue specific promoters useful in transgenic plants, including the cdc2a promoter and cyc07 promoter, will be known to those of skill in the art. (See for example, Ito, *et al., Plant Mol. Biol.*, 24:863, 1994; Martinez, *et al., Proc. Natl. Acad. Sci. USA*, 89:7360, 1992; Medford, *et al., Plant Cell*, 3:359, 1991; Terada, *et al., Plant Journal*, 3:241, 1993; Wissenbach, *et al., Plant Journal*, 4:411, 1993).

Optionally, a selectable marker may be associated with the nucleic acid sequence to be inserted. As used herein, the term "marker" refers to a gene encoding a trait or a phenotype which permits the selection of, or the screening for, a plant or plant cell containing the marker. Preferably, the marker gene is an antibiotic resistance gene whereby the appropriate antibiotic can be used to select for transformed cells from among cells that are not transformed. Examples of suitable selectable markers include adenosine deaminase, dihydrofolate reductase, hygromycin-B-phosphotransferase, thymidine kinase, xanthine-guanine phospho-ribosyltransferase and amino-glycoside 3'-O-phospho-transferase II (kanamycin, neomycin and G418 resistance). Other suitable markers will be known to those of skill in the art.

Although the following descriptions describe DET2, it is understood that this is an exemplary 5α-reductase and mammalian or other 5α-reductase are also included in the following descriptions. Vector(s) employed in the present invention for transformation of a plant cell include a nucleic acid sequence encoding DET2, operably associated with a promoter. To commence a transformation process in accordance with the present invention, it is first necessary to construct a suitable vector and properly introduce it into the plant cell. Details of the construction of vectors utilized herein are known to those skilled in the art of plant genetic engineering.

DET2 nucleic acid sequences utilized in the present invention can be introduced into plant cells using Ti plasmids of Agrobacterium tumefaciens, root-inducing (Ri) plasmids, and plant virus vectors. (For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9, and Horsch, *et al., Science,* 227:1229, 1985, both incorporated herein by reference). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of Agrobacterium, alternative methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, transformation using viruses or pollen and the use of microprojection.

One of skill in the art will be able to select an appropriate vector for introducing the DET2-encoding nucleic acid sequence in a relatively intact state. Thus, any vector which will produce a plant carrying the introduced DNA sequence should be sufficient. Even use of a naked piece of DNA would be expected to confer the properties of this invention, though at low efficiency. The selection of the vector, or whether to use a vector, is typically guided by the method of transformation selected.

The transformation of plants in accordance with the invention may be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology. (See, for example, Methods of Enzymology, Vol. 153, 1987, Wu and Grossman, Eds., Academic Press, incorporated herein by reference). As used herein, the term "transformation" means alteration of the genotype of a host plant by the introduction of DET2 nucleic acid sequence.

For example, a DET2 nucleic acid sequence can be introduced into a plant cell utilizing *Agrobacterium tumefaciens* containing the Ti plasmid, as mentioned briefly above. In using an *A. tumefaciens* culture as a transformation vehicle, it is most advantageous to use a non-oncogenic strain of Agrobacterium as the vector carrier so that normal non-oncogenic differentiation of the transformed tissues is possible. It is also preferred that the Agrobacterium harbor a binary Ti plasmid system. Such a binary system comprises 1) a first Ti plasmid having a virulence region essential for the introduction of transfer DNA (T-DNA) into plants, and 2) a chimeric plasmid. The latter contains at least one border region of the T-DNA region of a wild-type Ti plasmid flanking the nucleic acid to be transferred. Binary Ti plasmid systems have been shown effective to transform plant cells (De Framond, *Biotechnology*, 1:262, 1983; Hoekema, *et al., Nature*, 303:179, 1983). Such a binary system is preferred because it does not require integration into the Ti plasmid of Agrobacterium, which is an older methodology.

Methods involving the use of Agrobacterium in transformation according to the present invention include, but are not limited to: 1) co-cultivation of Agrobacterium with

cultured isolated protoplasts; 2) transformation of plant cells or tissues with Agrobacterium; or 3) transformation of seeds, apices or meristems with Agrobacterium.

In addition, gene transfer can be accomplished by *in planta* transformation by *Agrobacterium*, as described by Bechtold, *et al., (C.R. Acad. Sci. Paris*, 316:1194, 1993) and exemplified in the Examples herein. This approach is based on the vacuum infiltration of a suspension of *Agrobacterium* cells.

The preferred method of introducing DET2-encoding nucleic acid into plant cells is to infect such plant cells, an explant, a meristem or a seed, with transformed *Agrobacterium tumefaciens* as described above. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into plants.

Alternatively, DET2 encoding nucleic acid sequences can be introduced into a plant cell using mechanical or chemical means. For example, the nucleic acid can be mechanically transferred into the plant cell by microinjection using a micropipette. Alternatively, the nucleic acid may be transferred into the plant cell by using polyethylene glycol which forms a precipitation complex with genetic material that is taken up by the cell.

DET2 nucleic acid sequences can also be introduced into plant cells by electroporation (Fromm, *et al., Proc. Natl. Acad. Sci., U.S.A.*, 82:5824, 1985, which is incorporated herein by reference). In this technique, plant protoplasts are electroporated in the presence of vectors or nucleic acids containing the relevant nucleic acid sequences. Electrical impulses of high field strength reversibly permeabilize membranes allowing the introduction of nucleic acids. Electroporated plant protoplasts reform the cell wall, divide and form a plant callus. Selection of the transformed plant cells with the transformed gene can be accomplished using phenotypic markers as described herein.

Another method for introducing DET2 nucleic acid into a plant cell is high velocity ballistic penetration by small particles with the nucleic acid to be introduced contained either within the matrix of such particles, or on the surface thereof (Klein, *et al., Nature*

327:70, 1987). Bombardment transformation methods are also described in Sanford, *et al.* (*Techniques* 3:3-16, 1991) and Klein, *et al.* (*Bio/Techniques* 10:286, 1992). Although, typically only a single introduction of a new nucleic acid sequence is required, this method particularly provides for multiple introductions.

Cauliflower mosaic virus (CaMV) may also be used as a vector for introducing nucleic acid into plant cells (US Patent No. 4,407,956). CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid again may be cloned and further modified by introduction of the desired nucleic acid sequence. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

As used herein, the term "contacting" refers to any means of introducing DET2 into the plant cell, including chemical and physical means as described above. Preferably, contacting refers to introducing the nucleic acid or vector into plant cells (including an explant, a meristem or a seed), via *Agrobacterium tumefaciens* transformed with the DET2 encoding nucleic acid as described above.

Normally, a plant cell is regenerated to obtain a whole plant from the transformation process. The immediate product of the transformation is referred to as a "transgenote". The term "growing" or "regeneration" as used herein means growing a whole plant from a plant cell, a group of plant cells, a plant part (including seeds), or a plant piece (*e.g.*, from a protoplast, callus, or tissue part).

Regeneration from protoplasts varies from species to species of plants, but generally a suspension of protoplasts is first made. In certain species, embryo formation can then be induced from the protoplast suspension, to the stage of ripening and germination as natural embryos. The culture media will generally contain various amino acids and hormones, necessary for growth and regeneration. Examples of hormones utilized include auxins and cytokinins. It is sometimes advantageous to add glutamic acid and proline to the medium, especially for plant species such as corn and alfalfa. Efficient

regeneration will depend on the medium, on the genotype, and on the history of the culture. If these variables are controlled, regeneration is reproducible.

Regeneration also occurs from plant callus, explants, organs or parts. Transformation can be performed in the context of organ or plant part regeneration. (see *Methods in Enzymology*, Vol. 118 and Klee, *et al., Annual Review of Plant Physiology*, 38:467, 1987). Utilizing the leaf disk-transformation-regeneration method of Horsch, *et al.*, *Science*, 227:1229, 1985, disks are cultured on selective media, followed by shoot formation in about 2-4 weeks. Shoots that develop are excised from calli and transplanted to appropriate root-inducing selective medium. Rooted plantlets are transplanted to soil as soon as possible after roots appear. The plantlets can be repotted as required, until reaching maturity.

In vegetatively propagated crops, the mature transgenic plants are propagated by utilizing cuttings or tissue culture techniques to produce multiple identical plants. Selection of desirable transgenotes is made and new varieties are obtained and propagated vegetatively for commercial use.

In seed propagated crops, the mature transgenic plants can be self crossed to produce a homozygous inbred plant. The resulting inbred plant produces seed containing the newly introduced foreign gene(s). These seeds can be grown to produce plants that would produce the selected phenotype, *e.g.* increased yield.

Parts obtained from regenerated plant, such as flowers, seeds, leaves, branches, roots, fruit, and the like are included in the invention, provided that these parts comprise cells that have been transformed as described. Progeny and variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced nucleic acid sequences.

Plants exhibiting increased yield or biomass as compared with wild-type plants can be selected by visual observation. The invention includes plants produced by the method of the invention, as well as plant tissue and seeds.

In yet another embodiment, the invention provides a method for producing a genetically modified plant cell such that a plant produced from said cell produces increased yield as compared with a wild-type plant. The method includes contacting the plant cell with a DET2 nucleic acid sequence to obtain a transformed plant cell; growing the transformed plant cell under plant forming conditions to obtain a plant having increased yield. Conditions such as environmental and promoter inducing conditions vary from species to species, but should be the same within a species.

In another embodiment, the invention provides a method of producing a plant characterized as having increased yield by contacting a susceptible plant with a DET2 promoter-inducing amount of an agent which induces DET2 gene expression, wherein induction of DET2 gene expression results in production of a plant having increased yield as compared to a plant not contacted with the agent.

A "susceptible plant" refers to a plant that can be induced to utilize its endogenous DET2 gene to achieve increased yield. The term "promoter inducing amount" refers to that amount of an agent necessary to elevate DET2 gene expression above DET2 expression in a plant cell not contacted with the agent. For example, a transcription factor or a chemical agent may be used to elevate gene expression from DET2 native promoter, thus inducing the promoter and DET2 gene expression.

In another aspect of the invention, it is envisioned that increased expression of DET2 or other steroid 5α-reductases (e.g., mammalian) in a plant cell or in a plant, increases resistance of that cell/plant to plant pests or plant pathogens. For example, field studies have shown that brassinolides are effective as pesticides, therefore, increased expression of DET2 or other 5α-reductases would result in increased amounts of brassinolide in the plant. In addition, increased DET2 or other 5α-reductases expression may also cause increased resistance to pesticides (safeners). DET2 or other 5α-reductases therefore, protects plants against pests as well as against pesticides.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are

provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLES

Previously, *Arabidopsis* mutants that have characteristics of light-grown plants even when grown in the dark have been isolated. At least 11 such gene loci, known as *det, cop,* and *fus*, have been identified (Bowler and Chua, *Plant Cell*, 6:1529, 1994). Double-mutant analyses with several photoreceptor mutants suggest that *DET*1, COP1, and COP9 act in one signal transduction pathway, whereas *DET*2 acts in a different pathway (Bowler and Chua, *supra*). DET1, COP1, and COP9 all encode nuclear-localized proteins whose mode of action is not yet understood (Bowler and Chua, *supra*).

Loss-of-function mutations in *DET*2 have pleiotropic effects (Chory, *et al., ibid,* 3:445, 1991). In the dark, *det2* mutants are short, have thick hypocotyls, accumulate anthocyanins, have open, expanded cotyledons, and develop primary leaf buds. These morphological changes are accompanied by a 10-to 20-fold derepression of several light-responsive genes. In the light, *det2* mutants are smaller and darker green than wild type, show reduced cell size in the tissues examined (hypocotyl, cotyledons, and leaves), and have reduced apical dominance and male fertility. *det2* mutations also affect photoperiodic responses and cause a delay in flowering, a shortening of the circadian period of CAB (chlorophyl *a/b*-binding proteins) gene expression, inappropriate day and night regulation of gene expression, and a delay of leaf and chloroplast senescence (Chory, *et al., supra;* Chory, *et al., Plant physiol.*, 104:339, 1994; Millar, *et al., Science,* 267:1163, 1995). Such phenotype differences show that *DET*2 plays an important role throughout *Arabidopsis* development.

EXAMPLE 1

The *DET*2 gene was mapped to a 150-kb interval on *Arabidopsis* chromosome 2 (Fig. 1A). Figure 1 is a schematic illustration of the cloning and sequence analysis of the *DET*2

gene. Figure 1A shows a summary of positional cloning. Homozygous *det2*-1 mutant (Col-0) was crossed to either wild-type No-0 or La-er (geographic isolate designations).

DNA from F_2 *det*2 seedings was prepared (Deilaporta, *et al., Plant Mol. Biol. Rep.*, 1:19, 1983) for simple sequence length polymorphisms (SSLPs) (Bell and Ecker, *Genomic*, 19:137, 1994) and cleaved amplified polymorphic sequences (CAPS)(Konieczny and Ausubel, *Plant J.*, 4:403, 1993). Overlapping yeast artificial chromosome (YAC) clones were isolated from three separate YAC libraries of *Arabidopsis* (Ward and Jen, *Plant Mol. Biol.*, 14:561, 1990; J.R. Ecker, *Methods*, 1:186, 1990; Grill and Somerville, *Mol. Gen. Genet.*, 226:484, 1991) Fine-RFLP analysis was performed with F_2 *det*2 plants with recombination break points either in the m323-*DET*2 region (68 recombinants, two mapping populations) or in the *DET*2-nga 168 interval (31 recombinants).

Molecular marker nga168 was used as the starting point for identifying eight overlapping YAC clones covering ~800 kb of *Arabidopsis* genomic DNA. New CAPS markers were converted directly from YAC insert ends or derived from phage clones of an *Arabidopsis* genomic library isolated with YAC end probes. RFLP analysis delimited the *DET*2 locus to a 150 -kb region between the left ends of yUP2C12 and yUPSE10 in Arabidopsis chromosome 2.

A cosmid contig was assembled within this region from cosmid and phage clones isolated from two *Arabidopsis* genomic libraries (Olszewski, *et al., Nucleic Acids Res.*, 16:10765, 1988) by hybridization with yUP2C12, yUP6B10, YAC end probes, or cosmid-derived probes.

Cosmid DNAs were transformed into *det*2-1 plants by a modified vacuum infiltration method (Bechtold, *et al., Acad. Sci. Paris*, 316:1194, 1993; Bent, *et al., Science*, 265:1856, 1994) to identify cosmids containing the *DET*2 gene. Three cosmids, 2C12-19, 2C12-21, and 217-61, rescued det2 mutant phenotypes. A 20-kb genomic fragment was identified that can rescue the *det*2 phenotypes. Figure 2 shows a photo of light-grown 12-day-old seedlings (Figure 2A) and dark-grown 10-day-old seedlings (Figure 2B) after

complementation of *det*2 by the wild-type *DET*2 gene. (From left to right in each panel) Wild-type Col-0, *det*2-1, and transgenic *det*2-1 containing cosmid 217-61. Labeled Eco R1 fragments of cosmid 217-61 were used as probes to screen ~2 x 10^6 clones of an *Arabidopsis* complementary DNA (cDNA) library constructed in lambda ZAPII (Kieber, *et al., Cell,* 72:427, 1993). Positive clones were converted to plasmids by *in vivo* excision according to the manufacturer's protocol (Stratagene) and sequenced with gene-specific primers. The 20 kb fragment gives rise to at least three transcripts (Fig. 1A), one of which is altered in all *det*2 alleles analyzed and is derived from the *DET*2 gene (Fig. 1B).

The *DET*2 transcript contains a single, long open reading frame that encodes a 262-amino acid protein. The corresponding genomic sequences of eight *det*2 alleles, all which have similar mutant phenotypes, were determined. The transcribed region of the *DET*2 gene was amplified by polymerase chain reaction (PCR) from genomic DNAs of wild-type Col-0 and eight *det*2 alleles, subcloned into pGEM-T vector (Promega), and sequenced. To minimize PCR errors, at least four different clones from two independently amplified fragments were pooled for sequencing. Four alleles contain frame shifting deletions, and another two mutations cause premature termination of the *DET*2 protein. The two remaining alleles have a nonconservative substitution of lysine for glutamate at position 204 (Fig. 1B). Figure 1B shows a map of the gene structure of *DET*2 and mutations in the *DET*2 gene. Thick lines indicate exons, and the open box denotes an intron. Positions of mutations are relative to the initiation codon. Z, stop codon. Figure 1C shows the nucleotide and deduced amino acid sequence of DET2 (SEQ ID NO:1 and 2, respectively).

Figure 3 shows a sequence comparison of *DET*2 with mammalian steroid 5α-reductases. Figure 3A shows the deduced amino acid sequence of the *DET*2 gene aligned with the steroid 5α-reductases from rat (rS5R1 and rS5R2) and human (hS5R1 and hS5R2). Dashes indicate gaps introduced to maximize alignment, and residues conserved in at least two of the five sequences are shaded. Arrow indicates the glutamate mutated in *det*2-1 and *det*2-6 alleles. (Single-letter abbreviation for the amino acid residues are as

follows: A, Alta; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gin; R, Arg; S, Ser; T, The; V, Val; W, Trp; and Y, Tyr). Figure 3B shows a phylogenetic analysis of the relationship between *DET2* protein and mammalian steroid 5α-reductases. The scale measures the relative distance between sequences.

The deduced amino acid sequence of the *DET2* gene is similar to that of mammalian steroid 5α-reductases, with 38 to 42% sequence identity. (Database searches were performed at the U.S. National Center for Biotechnology information with the BLAST program (Altschul, *et al., J. Mol. Biol.*, 215:403, 1990). Sequence alignment and phylogenetic analysis were performed with the Megalign program (DNAStar) by the method of J. Hein (Higgins and Sharp, *Comput. Appl. Biosci.*, 5:151, 1989)). The sequence similarity increases to 54 to 60% when conservative substitutions are taken into account. Two isozymes (types 1 and 2) of steroid 5α-reductase have been isolated in rats and human (Wilson, *et al., Endocr. Rev.*, 14:577, 1993; Russell and Wilson, *Annu. Rev. Biochem.*, 63:25, 1994). Phylogenetic analysis shows that *DET2* is at least as closely related to type 2 enzymes as type 2 enzymes are related to type 1 enzymes (Fig. 3B).

Eighty percent of the absolutely conserved residues in mammalian enzymes are found in the predicted *DET2* protein. Mammalian steroid 5α-reductases catalyze the nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)-dependent conversion of testosterone to dihydrotestosterone, which is a key step in steroid metabolism and is essential for the embryonic development of male external genitalia and prostate (Wilson, *et al., supra*). The importance of this reaction is evident from certain hereditary forms of male pseudohermaphroditism in humans caused by steroid 5α-reductase deficiency. Sequences analysis of the steroid 5α-reductase type 2 gene from affected families identifies a missense mutation that causes a conservative substitution of aspartate for Glu^{197} (Wilson, *et al., supra*), corresponding to Glu^{204} in *DET2*. In the *det2*-1 and *det2*-6 alleles, this glutamate is changed to lysine, indicating that this glutamate has a similar critical function as in the human 5α-reductase. Because a conservative substitution at

this position causes inactivation of the human enzyme (Wilson, *et al., supra*), that the non-conservative glutamate-to-lysine change is predicted to completely abolish *DET2* activity. This could explain the severe phenotypes of the two missense alleles. Taken together, the data suggest that the *DET2* enzyme may catalyze a biochemical reaction similar to the reaction catalyzed by the human enzyme.

EXAMPLE 2

In plants, many steroids have been identified (J.M.C. Geuns, *Phytochemistry*, 17:1, 1978), but only brassinosteroids (BRs) have wide distribution throughout the plant kingdom and unique biological activity on plant growth when applied exogenously (N.B. Mandava, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 39:23, 1988; R.N. Arteca, (2), 206-213). A pathway for the biosynthesis of brassinolide, the most active BR, has recently been proposed on the basis of evidence from cell suspension cultures and whole-seeding experiments(Fujioka, *et al., Bio. Sci. Biotechnical. Biochem.*, 59:1973, 1995). Although the biosynthesis of brassinolide involves many oxidation steps, only two steps involve reduction. One occurs early in the pathway where a double bond in campesterol is reduced to form campestanol. This reaction is similar to that catalyzed by mammalian steroid 5α-reductases, suggesting that *DET2* could catalyze the conversion of campesterol to campestanol. Because the *Arabidopsis* genome does not contain any other sequences that are closely related to *DET2*, one possibility is that the *det2* phenotype is due to reduction or elimination of BR biosynthesis.

To test this hypothesis, *det2* seedlings were treated with exogenous brassinolide. Figure 4A shows the proposed function of *DET2* protein in the brassinolide biosynthetic pathway. Asterisk (*) indicates six intermediate steps (Fujicka, *et al., supra*). Figure 4B shows dark-grown 10-day-old seedlings and Figure 4C light-grown 12-day-old seedlings. Wild-type, det2-1, and brassionolide-treated *det2*-1 plants are depicted from left to right in each panel. Seeds were germinated on moist Whatman papers placed on MS medium (0.5xMS salts(Gibco), 1x Gamborg's B5 vitamins (Sigma), 0.8% phytagar, and 1%

sucrose pH 5.7) for 2 days and transferred to fresh plates supplemented with various concentrations of auxin (IAA, 0 to 10^{-5} M), brassinolide (0 to 10^{-6} M), and gibberellins (GA1 and GA4, 0 to 10^{-5} M). Hormones were sterile-filtered into the cooling MS medium. For dark-grown seedlings, seeds were exposed to 2-hour light treatment before their plates were wrapped with three layers of aluminum foil and the seedlings were transferred under a green safe-light. The hypocotyl lengths of 10-day-old etiolated seedlings and 12-day-old light-grown wild-type plants were measured.). Figure 4D shows a dose-response of brassinolide-induced hypocotyl elongation of dark-grown seedlings and light-grown wild-type). Data represent the mean ± SE obtained from triplicate determinations, each with an average sample size of 12 seedlings.

Although addition of brassinolide at 10^{-6} M to the growth medium had no effect on wild-type seedlings in the dark, the short hyocotyl phenotype of dark-grown *det2* seedlings was rescued (Fig. 4, B and D). Similarly when added at 10^{-7} M, brassinolide had no effect on the petioles and leaves of wild-type seedlings but fully suppressed the dwarf phenotypes of these organs in light-grown det2 plants (Fig. 4C). In contrast, neither applied gibberellins (GA1 or GA4, 10^{-8} to 10^{-5} M) nor auxins (1A, A 10^{-6} and 10^{-5} M) rescued the *det2* defects. Brassinolide treatment reversed the inhibition of hypocotyl elongation caused either by *det1* mutation or light (FIG. 4D), but it did not complement the mutant phenotypes of either dark-or light-grown *det1* seedlings, supporting previous genetic studies that *DET*1 and *DET*2 act on separate pathways controlling light-regulated processes.

EXAMPLE 3

Arabidopsis det2 mutants are small dark-green dwarfs displaying pleiotropic defects in light-regulated development during multiple stages of the plant life cycle. The *DET2* gene encodes a protein that shares ~40% sequence identity with mammalian steroid 5α-reductases and is implicated in the synthesis of a class of plant steroids, the brassinosteroids. The following example shows that the DET2 protein, when expressed in human embryonic kidney 293 cells, catalyzes the 5α-reduction of several animal steroid substrates and has similar kinetic properties to the mammalian steroid 5α-reductase enzymes. Moreover, human steroid 5α-reductases expressed in *det2* mutant plants can substitute for DET2 in brassinosteroid biosynthesis. These data indicate that DET2 is an ortholog of the mammalian steroid 5α-reductases and provide further evidence that brassinosteroids play an essential role in light-regulated plant development. Figure 5 shows the chemical reactions catalyzed by mammalian steroid 5α-reductases (A) and Arabidopsis DET2 (B).

1. Plant materials and growth conditions:

The standard wild-type genotype used was Arabidopsis thaliana Columbia (Col-0). The det2-1 mutant used in this study has been described (Chory, J., Nagpal, P. & Peto, C.A. (1991) *Plant Cell* 3, 445-459). Seeds were surfaced sterilized by washing for 1 min in 95% ethanol, followed by 12 min in a 1:3 dilution of bleach (Clorox) containing 0.02% (v/v) Tween-20. The seeds were then washed 3 times with sterilized distilled water, and resuspended in 0.08% phytagar (Gibco BRL). After treatment for 2 days at 4°C to induce germination, seeds were sown in Petri plates containing 0.5X MS (Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* 15, 473-497) medium (pH 5.7) supplemented with 1% sucrose, 0.8% phytagar, and 1X Gamborg's B5 vitamins (Sigma) and maintained in growth chambers at 21°C with a 16-hr photoperiod. Plants were grown in the dark by wrapping plates with three layers of aluminum foil. For all experiments, det2 and the

Col-0 wild-type plants were grown side-by-side under the same light and humidity conditions.

2. Expression of DET2 cDNA in human embryonic kidney 293 Cells:

A 948-bp fragment from a DET2 cDNA clone containing 18 bp of 5'-untranslated sequence, an open reading frame of 786 bp and 154 bp of 3'-untranslated sequence was ligated into the pCMV5 expression vector (Anderson, S., Davis, D.L., Dahlback, H., Jornvall, H & Russell, D.W. (1989) *J. Biol. Chem.*, 264, 8222-8229). As a control, a mutant det2 protein was also expressed in the pCMV5 vector (pCMV5-det2-1). This construct, which contains a single nucleotide mutation (G-A) that changes Glu204 to Lys204, was made by replacing the wild-type DET2 sequence with the same restriction fragment derived from PCR amplified det2-1 genomic DNA. The resulting plasmid was then digested with EcoRI/BglII and cloned into the pCMV5 expression vector. Five mg of expression plasmid containing either wild-type DET2 or mutated det2-1 cDNA was transfected into human embryonic kidney 293 cells (ATCC CRL1573) by a calcium phosphate precipitation procedure as described previously (Normington, K. & Russell, D.W. (1992) *J. Biol. Chem.* 267, 19548-19554). Sixteen hours after transfection, steroid 5α-reductase activity was assayed in either intact cells or cell lysates as described (Anderson, S., Bishop, R.W. & Russell, D.W. (1989) *J. Biol. Chem.* 264, 16249-16255; Thigpen, A.E. & Russell, D.W. (1992) *J. Biol. Chem.* 267, 8577-8583).

3. Transformation of det2-1 mutants.

A human cDNA encoding either the type 1 or type 2 steroid 5α-reductase was cloned into the pMD1 vector, a derivative of pBI121 (Clontech), which contains the cauliflower mosaic virus 35S promoter (CaMV) and a nopaline synthase transcriptional terminator (Nos-ter), to generate pMD1-hS5R (human steroid 5α-reductase) plasmids (Fig. 8A). Agrobacterium strain GV3101, transformed with a pMD1-hS5R construct, was used to

transform det2-1 mutants by the vacuum infiltration method as described previously (Bechtold, N., Ellis, J. & Pelletier, G. (1993) *C.R. Acad. Sci. Paris* 316, 1188-1193). The transformants (T1) were selected on 0.5X MS medium (pH 5.7) (*supra*) supplemented with 1% sucrose, 0.8% phytagar, 1X Gamborg's B5 vitamins, and 25 mg/ml kanamycin. Kanamycin-resistant seedlings were transferred to soil, maintained at 23°C under a 16-hr-light and 8-hr-dark cycle, allowed to self-pollinate, and T2 seeds were collected.

4. Treatment of etiolated seedlings with 4-MA.

Seeds were germinated on 0.5X MS (pH 5.7) medium (*supra*) supplemented with 1% sucrose, 1X Gamborg's B5 vitamins, 0.8% phytagar, and varying concentrations of 4-MA (17b-(N,N-diethyl)carbamoyl-4-methyl-4-aza-5a-androstan-3-one, a gift from Merck Sharp & Dohme Research Laboratories). Following a 2-hour light treatment, plates were wrapped with three layers of aluminum foil and kept at 21°C in a growth chamber. The hypocotyl lengths of 10-day-old etiolated seedlings were measured.

5. DNA and RNA analyses.

Arabidopsis DNA was isolated as described previously (Li, J. & Chory, J., (1996) in *Methods in Molecular Biology: Arabidopsis Protocols*, eds. Martinez-Zapater, J.M. & Sallnas, J. (Humana, ••, NJ), in press). For amplification of a genomic DET2 fragment, 1 ml (10-20 ng) of plant DNA was used as a template in a 50 ml reaction mixture containing 5 ml of 10X Taq polymerase buffer (Stratagene), 200 mM deoxynucleoside triphosphates (dNTPs), 125 ng each of forward and reverse primers, and 2.5 units of Taq polymerase. Amplification reactions were conducted in a thermocycler (ERICOMP) by denaturing the template DNA for 10 min at 95°C followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, extension at 72°C for 90 sec, and a final extension period of 10 minutes at 72°C. For amplifying human steroid 5 -reductase cDNAs in transgenic det2-1 plants, 10% DMSO was added in the PCR reaction (to overcome the problem caused by the high GC content of the cDNAs), together with

primers derived from the CaMV 35S promoter and each of the cDNAs. Total RNA was isolated from 2-week-old seedlings by the method of Napoli et al. (Napoli, C., Lemieux, C. & Jorgensen, R. (1990) *Plant Cell* 2, 279-289) and RNA gel blot hybridizations were performed as described previously (Chory, J., Nagpal, P. & Peto, C.A. (1991) *Plant Cell* 3, 445-459).

To determine whether the Arabidopsis DET2 locus encodes a functional steroid 5α-reductase, a full length DET2 cDNA was cloned into a mammalian expression vector, pCMV5 (*supra*) and introduced into cultured human embryonic kidney 293 cells by a CaPO4-mediated transfection protocol (*supra*). Sixteen hours after transfection, radiolabeled steroids were added to the cell medium and their conversion to 5α-reduced forms was monitored by thin layer chromatography (Normington, K. & Russell, D.W. (1992) *J. Biol. Chem.* 267, 19548-19554). As shown in Fig. 6, cells transfected with a pCMV5 expression vector lacking a cDNA insert displayed no measurable steroid 5α-reductase activity, while introduction of a pCMV5 expression vector containing a cDNA of either human type 1 steroid 5α-reductase or the Arabidopsis DET2 resulted in reduction of radiolabeled progesterone to 4,5-dihydroprogesterone. Consistent with our previous prediction (Li, J., Nagpal, P., Vitart, V., McMorris, C.T. & Chory, J. (1996) *Science* 272, 398-401), the Glu204Lys mutation of det2-1 totally inactivated the steroid 5α-reductase activity of DET2, as 293 cells transfected with det2-1 cDNA failed to convert the radiolabeled progesterone to its 5α-reduced form (Fig. 6).

Like mammalian steroid 5α-reductases (Russell, D.W. & Wilson, J.D. 1994 *Annu. Rev. Biochem.*, 63, 25-61), Arabidopsis DET2 can catalyze the 5α-reduction of several steroids with a 3-oxo-D4,5 structure, including testosterone and androstenedione. Since the hypothesized substrates of Arabidopsis DET2 in BR biosynthesis is campesterol or its analogs (sitosterol or stigmasterol), which contain a 3b-hydroxyl-D5,6 structure, we measured the steroid 5α-reductase activity of DET2 towards several radiolabeled steroids with this structure including cholesterol, pregnenolone, and dehydroepiandrosterone. In

line with the results obtained with mammalian steroid 5α-reductases (Hsia, S.L. & Voigt, W. (1974) *J. Invest. Dermatol.* 62, 224-227), the 293 cells transfected with a cDNA of either the human type 1 steroid 5α-reductase or DET2 failed to 5α-reduce these substrates (data not shown), implying that plants require the presence of an additional enzyme to convert campesterol to 3-oxo-D4,5 -campesterol before 5α-reduction by the DET2 enzyme.

Kinetic properties of expressed DET2 protein. An in vitro assay (*supra*) was used to study the enzyme kinetics of the kidney 293 cell-expressed DET2 protein. The apparent Km value for testosterone was determined to be 2.5 mM with a Vmax of 0.2 nmol/(min-1•mg-1) (Fig. 7A), while the apparent Km value for progesterone was 0.4 mM with a Vmax of 0.5 nmol/(min-1•mg-1) (Fig. 7B). These values compare favorably with those calculated for the human type 1 isozyme (Km = 1.7 mM for testosterone and Km = 1.3 mM for progesterone). Like the mammalian enzymes, the steroid 5α-reductase activity of DET2 requires NADPH rather than NADH as a cofactor and the catalyzed reaction was irreversible.

4-Azasteroids are a class of selective and potent inhibitors of mammalian steroid 5α-reductase isozymes. To determine if the DET2 enzyme was also inhibited by these drugs, cell lysates containing recombinant DET2 were incubated with progesterone as substrate and various amounts of the 4-azasteroid, 4-MA. Increasing the concentration of 4-MA in the reaction mixture progressively inhibited DET2 enzyme activity (Fig. 7C). Inspection of the data revealed a competitive mode of inhibition with a calculated apparent Ki of 300 nM. In experiments not shown, two other inhibitors of mammalian steroid 5α-reductase isozymes, finasteride (a 4-azasteroid) and LY191704 (a non-steroidal benzoquinolinone), did not affect the activity of recombinant DET2.

The mammalian steroid 5α-reductase type 1 and type 2 isozymes are distinguished by their pH optima. In all species so far examined, the type 1 isozyme has an alkaline pH optimum (Vmax at about pH 8.0,) and the type 2 isozyme has an acidic pH optimum

(Vmax at pH 5.0-5.5) (*supra*). The pH optimum of the plant enzyme was measured to determine which mammalian isozyme the DET2 enzyme most closely resembled with respect to this biochemical parameter. The data of Fig. 7D show that the pH versus enzyme activity curve obtained with cell lysates containing recombinant DET2 protein was symmetrical with an optimum at pH 6.8, which is between the pH optima of the two mammalian isozymes.

Human steroid 5α-reductase cDNAs complement the plant det2-1 mutation. If DET2 is a functional homolog of human steroid 5α-reductases, then expression of one or the other human enzyme should complement det2 mutation in plants and rescue the mutant phenotypes. To test this hypothesis, we stably introduced cDNAs encoding either the type 1 or type 2 human steroid 5α-reductase into det2-1 mutants using Agrobacterium-mediated transformation (*supra*). In these experiments, two constructs with either a full-length cDNA or a cDNA with a truncated 3'-untranslated region were prepared for each type of human steroid 5α-reductase cDNA. These were cloned into a binary vector, pMD1, which contains a cauliflower mosaic virus 35S promoter and a nopaline synthase 3'-untranslated sequence (Fig 8A).

In one set of transformation experiments, 64 transgenic plants were obtained. Among these, 57 displayed wild-type phenotypes and a remaining 7 lines displayed intermediate phenotypes between det2-1 and wild-type plants (Table 1). As shown in Fig. 8B-8D, the presence of either human steroid 5α-reductase cDNA in the det2-1 mutant background rescued both the dark and light phenotypes of the mutation. All 64 transgenic lines were tested for the insertion of a human cDNA into their genomes by PCR using oligonucleotide primers derived from the CaMV 35S promoter and a human 5α-reductase cDNA. PCR products of the expected size for either type of human steroid 5α-reductase cDNA were amplified from the genomic DNAs of all transgenic plants (summarized in Table 1), while nontransformed controls of wild-type or det2-1 heterozygous and

homozygous plants did not yield positive amplification signals. Since the det2-1 mutation causes a G-A transition, leading to the elimination of an MnlI restriction site, an MnlI restriction fragment length polymorphism (RFLP) exists between det2-1 mutants and wild-type plants. A PCR-based RFLP analysis was used to confirm that each of the 64 transgenic lines still contained the det2-1 mutation (Table 1), thereby eliminating the possibility that the observed phenotypic normalization was due to an accidental introduction of a wild-type DET2 gene into their genomes or to reversion at the mutated site.

Table 1

Summary of transgenic *det2-1* plants carrying human steroid 5α-reductase cDNAs

	Number of plants			
Constructs	Showing kanamycin resistance	Containing hS5R cDNA	Having *det2-1* mutation	Displaying wild-type phenotype
hS5R1-1.3kb	8	8	8	8
hS5R1-2.1kb	18	18	18	18
hS5R2-0.8kb	18	18	18	16
hS5R2-2.4kb	20	20	20	15

Transgenic plants of each construct were obtained from two independent transformation experiments. See above description and Figure 8A for a description of constructs.

To prove that the observed phenotypic normalization was caused by expression of a human steroid 5α-reductase cDNA in the transgenic plants, germinated seeds were collected from two representative transgenic lines, containing either a type 1 or a type 2 human cDNA, on a synthetic growth medium with different concentrations of 4-MA, an inhibitor of the mammalian steroid 5α-reductases. Fig. 9 shows the effect of increasing concentrations of 4-MA on the hypocotyl elongation of dark-grown plants. While increasing concentrations of 4-MA had little effect on the hypocotyl elongation of either wild-type or det2-1 seedlings, the drug caused a significant decrease of hypocotyl growth in the two transgenic lines. The differential effects of 4-MA on det2-1, wild-type and the two transgenic lines observed in these experiments were consistent with the biochemical properties of the individual human steroid 5α-reductases and DET2. The Ki for 4-MA of the human steroid 5α-reductase type 1 (8.0 nM) is twice that of the type 2 enzyme (4.0 nM) (*supra*), while the Ki for 4-MA of DET2 is at least 30 fold higher (300 nM) than those of the human isozymes (Fig. 7C).

The second line of evidence that expression of human steroid 5α-reductase cDNAs rescued det2-1 mutant phenotypes came from observations that the progeny of some of the individual transformants looked more similar to det2-1 mutants than to wild-type plants, although their parental lines exhibited a fully wild-type phenotype. We suspected that this phenomenon was due to loss of transgene expression in the T2 generation, which has been previously observed in various transgenic lines with the pMD1 binary vector . Therefore, the expression levels of the human cDNAs were examined in several transgenic lines by RNA blotting. The steady-state level of human steroid 5α-reductase mRNA expressed in the transgenic det2-1 plants (Fig. 10B) was correlated with the degree of mutant rescue as indicated by both the hypocotyl length of the dark-grown seedlings (Fig. 10A) and the overall morphology of light-grown plants (Fig. 10C). Plants showing the highest level of human transcripts among all lines examined exhibited full

wild-type stature in the light and their hypocotyls were even longer than those of wild-type controls in the dark. In contrast, the lines showing the lowest levels of human mRNAs looked more like det2-1 mutants than wild-type plants in the light and had intermediate hypocotyl lengths in the dark. Based on these results, we concluded that the human cDNAs can rescue det2-1 defects and that the expression of human steroid 5α-reductase cDNAs in the det2-1 transgenic plants correlated with the degree of rescue of their mutant phenotypes.

EXAMPLE 4

Rice was transformed with two constructs, one which allowed for DET2 overexpression and one for underexpression (antisense). Standard Agrobacterium expression vectors were used, in which the DET2 coding sequence was expressed in reverse orientation from the cauliflower mosaic virus 35S promoter (Metzlaff et al. *Cell* 88:845, 1997). The antisense plants had phenotypes similar to det2 mutants, *i.e.*, they were small with reduced male fertility and reduced apical dominance. The lines with moderate overexpression were larger than wild type and based on a few lines had an increase in yield of about 15% (the mass of seeds is 15% more than wild type seeds). High overexpressers were not healthy, which is consistent with the idea that too much of the hormone is detrimental.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Salk Institute for Biological Studies et al.

(ii) TITLE OF INVENTION: NOVEL PLANT STEROID 5 alpha REDUCTASE, DET2

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
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(F) ZIP: 92037

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/634,475
(B) FILING DATE: 18-APR-1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Haile, Lisa A.
(B) REGISTRATION NUMBER: 38,347
(C) REFERENCE/DOCKET NUMBER: 07251/015001

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 619/678-5070
(B) TELEFAX: 619/678-5099

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 974 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTCCATAA CCCGAAAAAT GGAAGAAATC GCCGATAAAA CCTTCTTCCG ATACTGTCTC 60

CTCACTCTTA TTTTCGCCGG CCCACCAACC GCCGTCCTTC TGAAATTCCT CCAAGCTCCT 120

TACGGTAAAC ACAACCGTAC CGGATGGGGT CCCACCGTAT CTCCACCGAT TGCTTGGTTC 180

GTCATGGAGA GCCCAACCTT GTGGCTCACT CTCCTCCTCT TCCCCTTTGG TCGTCACGCT 240

CTCAACCCTA AATCTCTACT TCTATTCTCT CCTTATCTCA TTCATTACTT CCACCGCACC 300

ATCATTTACC CTCTTCGCCT CTTCCGCAGC TCCTTCCCCG CCGGTAAAAA CGGATTTCCG 360

ATCACCATCG CCGCCTTGGC TTTCACCTTT AATCTCCTCA ATGGTTATAT CCAGGCGAGG 420

TGGGTTTCGC ATTACAAGGA TGACTACGAA GACGGAAACT GGTTCTGGTG GCGGTTTGTT 480

ATCGGTATGG TGGTTTTCAT AACCGGCATG TATATAAATA TCACGTCGGA CCGCACTTTG 540

GTACGATTGA AGAAAGAGAA CCGGGGAGGT TATGTGATAC CGAGAGGAGG CTGGTTCGAG 600

TTGGTAAGCC GTCCGAATTA TTTTGGAGAG GCGATTGAGT GGTTGGGCTG GGCTGTTATG 660

ACTTGGTCTT GGGCCGGTAT TGGATTTTTT CTGTACACGT GTTCCAATTT GTTTCCGCGT 720

GCACGTGCGA GTCACAAGTG GTACATTGCC AAGTTCAAGG AAGAGTATCC CAAGACTCGT 780

AAAGCTGTTA TTCCTTTTGT GTACTGAGAA TTGAGAAAGT TGAAAACTAG TTTATCATAT 840

GTTATGTGTC AATTTGTTTC CAAACTACCT TTGTCAAAAT TTCCAGTAAC CGGTTTAATT 900

CCAACACGGT TTAGATCTTA TGTTGGTATC TTCAACAATG CACAACAAAC TGTGTATTCT 960

TTAGACAAAT TTTA 974

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 262 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Glu Ile Ala Asp Lys Thr Phe Phe Arg Tyr Cys Leu Leu Thr
1 5 10 15

Leu Ile Phe Ala Gly Pro Pro Thr Ala Val Leu Leu Lys Phe Leu Gln
20 25 30

Ala Pro Tyr Gly Lys His Asn Arg Thr Gly Trp Gly Pro Thr Val Ser
35 40 45

Pro Pro Ile Ala Trp Phe Val Met Glu Ser Pro Thr Leu Trp Leu Thr
50 55 60

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Leu Leu Leu Phe Pro Phe Gly Arg His Ala Leu Asn Pro Lys Ser Leu
65                  70                  75                  80

Leu Leu Phe Ser Pro Tyr Leu Ile His Tyr Phe His Arg Thr Ile Ile
                85                  90                  95

Tyr Pro Leu Arg Leu Phe Arg Ser Ser Phe Pro Ala Gly Lys Asn Gly
            100                 105                 110

Phe Pro Ile Thr Ile Ala Ala Leu Ala Phe Thr Phe Asn Leu Leu Asn
        115                 120                 125

Gly Tyr Ile Gln Ala Arg Trp Val Ser His Tyr Lys Asp Asp Tyr Glu
    130                 135                 140

Asp Gly Asn Trp Phe Trp Trp Arg Phe Val Ile Gly Met Val Val Phe
145                 150                 155                 160

Ile Thr Gly Met Tyr Ile Asn Ile Thr Ser Asp Arg Thr Leu Val Arg
                165                 170                 175

Leu Lys Lys Glu Asn Arg Gly Gly Tyr Val Ile Pro Arg Gly Gly Trp
            180                 185                 190

Phe Glu Leu Val Ser Arg Pro Asn Tyr Phe Gly Glu Ala Ile Glu Trp
        195                 200                 205

Leu Gly Trp Ala Val Met Thr Trp Ser Trp Ala Gly Ile Gly Phe Phe
    210                 215                 220

Leu Tyr Thr Cys Ser Asn Leu Phe Pro Arg Ala Arg Ala Ser His Lys
225                 230                 235                 240

Trp Tyr Ile Ala Lys Phe Lys Glu Glu Tyr Pro Lys Thr Arg Lys Ala
                245                 250                 255

Val Ile Pro Phe Val Tyr
            260
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 246 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Leu Ile Phe Ala Gly Pro Pro Thr Ala Val Leu Leu Lys Phe Leu Gln
1               5                   10                  15
```

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Ala Pro Tyr Gly Lys His Asn Arg Thr Gly Trp Gly Pro Thr Val Ser
            20                  25                  30

Pro Pro Ile Ala Trp Phe Val Met Glu Ser Pro Thr Leu Trp Leu Thr
        35                  40                  45

Leu Leu Leu Leu Pro Phe Gly Arg His Ala Leu Asn Pro Lys Ser Leu
    50                  55                  60

Leu Leu Phe Ser Pro Tyr Leu Ile His Tyr Phe Leu Arg Thr Ile Ile
65                  70                  75                  80

Tyr Pro Leu Arg Leu Phe Arg Ser Ser Phe Pro Ala Gly Lys Asn Gly
                85                  90                  95

Phe Pro Ile Thr Ile Ala Ala Leu Ala Phe Thr Phe Asn Leu Leu Asn
            100                 105                 110

Gly Tyr Ile Gln Ala Arg Trp Val Ser His Tyr Lys Asp Asp Tyr Glu
        115                 120                 125

Asp Gly Asn Trp Phe Trp Trp Arg Phe Val Ile Gly Met Val Val Phe
    130                 135                 140

Ile Thr Gly Met Tyr Ile Asn Ile Thr Ser Asp Arg Thr Leu Val Arg
145                 150                 155                 160

Leu Lys Lys Glu Asn Arg Gly Gly Tyr Val Ile Pro Arg Gly Gly Trp
                165                 170                 175

Phe Glu Leu Val Ser Arg Pro Asn Tyr Phe Gly Glu Ala Ile Glu Trp
            180                 185                 190

Leu Gly Trp Ala Val Met Thr Trp Ser Trp Ala Gly Ile Gly Phe Phe
        195                 200                 205

Leu Tyr Thr Cys Ser Asn Leu Phe Pro Arg Ala Arg Ala Ser His Lys
    210                 215                 220

Trp Tyr Ile Ala Lys Phe Lys Glu Glu Tyr Pro Lys Thr Arg Lys Ala
225                 230                 235                 240

Val Ile Pro Glu Val Tyr
                245
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 239 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Gly Phe Met Ala Phe Val Ser Ile Val Gly Leu Arg Ser Val Gly Ser
1               5                  10                  15

Pro Tyr Gly Arg Tyr Ser Pro Gln Trp Pro Gly Ile Arg Val Pro Ala
            20                  25                  30

Arg Pro Ala Trp Phe Ile Gln Glu Leu Pro Ser Met Ala Trp Pro Leu
        35                  40                  45

Tyr Glu Tyr Ile Arg Pro Ala Ala Ala Arg Leu Gly Asn Leu Pro Asn
    50                  55                  60

Arg Val Leu Leu Ala Met Phe Leu Ile His Tyr Val Gln Arg Thr Leu
65                  70                  75                  80

Val Phe Pro Val Leu Ile Arg Gly Gly Lys Pro Thr Leu Leu Val Thr
                85                  90                  95

Phe Val Leu Ala Phe Leu Phe Cys Thr Phe Asn Gly Tyr Val Gln Ser
            100                 105                 110

Arg Tyr Leu Ser Gln Phe Ala Val Tyr Ala Glu Asp Trp Val Thr His
        115                 120                 125

Pro Cys Phe Leu Thr Gly Phe Ala Leu Trp Leu Val Gly Met Val Ile
    130                 135                 140

Asn Ile His Ser Asp His Ile Leu Arg Asn Leu Arg Lys Pro Gly Glu
145                 150                 155                 160

Thr Gly Tyr Lys Ile Pro Arg Gly Gly Leu Phe Glu Tyr Val Ser Ala
                165                 170                 175

Ala Asn Tyr Phe Gly Glu Leu Val Glu Trp Cys Gly Phe Ala Leu Ala
            180                 185                 190

Ser Trp Ser Leu Gln Gly Val Val Phe Ala Leu Phe Thr Leu Ser Thr
        195                 200                 205

Leu Leu Thr Arg Ala Lys Gln His His Gln Trp Tyr His Glu Lys Phe
    210                 215                 220

Glu Asp Tyr Pro Lys Ser Arg Lys Ile Leu Ile Pro Phe Val Leu
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 243 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ala Tyr Leu Gln Cys Ala Val Gly Cys Ala Val Phe Ala Arg Asn Arg
1               5                  10                  15

Gln Thr Asn Ser Val Tyr Gly Arg His Ala Leu Pro Ser His Arg Leu
            20                  25                  30

Arg Val Pro Ala Arg Ala Ala Trp Val Val Gln Glu Leu Pro Ser Leu
        35                  40                  45

Ala Leu Pro Leu Tyr Gln Tyr Ala Ser Glu Ser Ala Pro Arg Leu Arg
    50                  55                  60

Ser Ala Pro Asn Cys Ile Leu Leu Ala Met Phe Leu Val His Tyr Gly
65                  70                  75                  80

His Arg Cys Leu Ile Tyr Pro Phe Leu Met Arg Gly Gly Lys Pro Met
                85                  90                  95

Pro Leu Leu Ala Cys Thr Met Ala Ile Met Phe Cys Thr Cys Asn Gly
            100                 105                 110

Tyr Leu Gln Ser Arg Tyr Leu Ser His Cys Ala Val Tyr Ala Asp Asp
        115                 120                 125

Trp Val Thr Asp Pro Arg Phe Leu Ile Gly Phe Gly Leu Trp Leu Thr
    130                 135                 140

Gly Met Leu Ile Asn Ile His Ser Asp His Ile Leu Arg Asn Leu Arg
145                 150                 155                 160

Lys Pro Gly Asp Thr Gly Tyr Lys Ile Pro Arg Gly Gly Leu Phe Glu
                165                 170                 175

Tyr Val Thr Ala Ala Asn Tyr Phe Gly Glu Ile Met Glu Trp Cys Gly
            180                 185                 190

Tyr Ala Leu Ala Ser Trp Ser Val Gln Gly Ala Ala Phe Ala Phe Phe
        195                 200                 205

Thr Phe Gly Phe Leu Ser Gly Arg Ala Lys Glu His His Glu Trp Tyr
    210                 215                 220

Leu Arg Lys Phe Glu Glu Tyr Pro Lys Phe Arg Lys Ile Ile Ile Pro
225                 230                 235                 240

Phe Leu Phe
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 238 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Ala Thr Met Gly Thr Leu Ile Leu Cys Leu Gly Lys Pro Ala Ser
1 5 10 15

Tyr Gly Lys His Thr Glu Ser Val Ser Ser Gly Val Pro Phe Leu Pro
20 25 30

Ala Arg Ile Ala Trp Phe Leu Gln Glu Leu Pro Ser Phe Val Val Ser
35 40 45

Val Gly Met Leu Ala Trp Gln Pro Arg Ser Leu Phe Gly Pro Pro Gly
50 55 60

Asn Val Leu Leu Ala Leu Phe Ser Ala His Tyr Phe His Arg Thr Phe
65 70 75 80

Ile Tyr Ser Leu Leu Thr Arg Gly Arg Pro Phe Pro Ala Val Leu Phe
85 90 95

Leu Arg Ala Thr Ala Phe Cys Ile Gly Asn Gly Leu Leu Gln Ala Tyr
100 105 110

Tyr Leu Val Tyr Cys Ala Glu Tyr Pro Glu Glu Trp Tyr Thr Asp Val
115 120 125

Arg Phe Ser Phe Gly Val Phe Leu Phe Ile Leu Gly Met Gly Ile Asn
130 135 140

Ile His Ser Asp Tyr Thr Leu Arg Gln Leu Arg Lys Pro Gly Glu Val
145 150 155 160

Ile Tyr Arg Ile Pro Arg Gly Gly Leu Phe Thr Tyr Val Ser Gly Ala
165 170 175

Asn Phe Leu Gly Glu Ile Ile Glu Trp Ile Gly Tyr Ala Leu Ala Thr
180 185 190

Trp Ser Val Pro Ala Phe Ala Phe Ala Phe Phe Thr Leu Cys Phe Leu
195 200 205

Gly Met Gln Ala Phe Tyr His His Arg Phe Tyr Leu Lys Met Phe Lys
210 215 220

Asp Tyr Pro Lys Ser Arg Lys Ala Leu Ile Pro Phe Ile Phe
225 230 235

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Val Ala Leu Gly Ala Leu Ala Leu Tyr Val Ala Lys Pro Ser Ser
1 5 10 15

Ser Ser Gly Tyr Gly Lys His Thr Glu Ser Leu Lys Pro Ala Ala Thr
20 25 30

Arg Leu Pro Ala Arg Ala Ala Trp Phe Leu Gln Glu Leu Pro Ser Phe
35 40 45

Ala Val Pro Ala Gly Ile Leu Ala Arg Gln Pro Leu Ser Leu Phe Gly
50 55 60

Pro Pro Gly Thr Val Leu Leu Gly Leu Phe Cys Val His Tyr Phe His
65 70 75 80

Arg Thr Phe Val Tyr Ser Leu Leu Asn Arg Gly Arg Pro Tyr Pro Ala
85 90 95

Ile Leu Ile Leu Arg Gly Thr Ala Phe Cys Thr Gly Asn Gly Val Leu
100 105 110

Gln Gly Tyr Tyr Leu Ile Tyr Cys Ala Glu Tyr Pro Asp Gly Trp Tyr
115 120 125

Thr Asp Ile Arg Phe Ser Leu Gly Val Phe Leu Phe Ile Leu Gly Met
130 135 140

Gly Ile Asn Ile His Ser Asp Tyr Ile Leu Arg Gln Leu Arg Lys Pro
145 150 155 160

Gly Glu Ile Ser Tyr Arg Ile Pro Gln Gly Gly Leu Phe Thr Tyr Val
165 170 175

Ser Gly Ala Asn Phe Leu Gly Glu Ile Ile Glu Trp Ile Gly Tyr Ala
180 185 190

Leu Ala Thr Trp Ser Leu Pro Ala Leu Ala Phe Ala Phe Phe Ser Leu
195 200 205

Cys Phe Leu Gly Leu Arg Ala Phe His His His Arg Phe Tyr Leu Lys
210 215 220

Met Phe Glu Asp Tyr Pro Lys Ser Arg Lys Ala Leu Ile Pro Phe Ile
225 230 235 240

Phe

What is claimed is:

1. A substantially purified DET2 polypeptide.

2. The polypeptide according to claim 1, wherein DET2 is characterized as:
   a) having a molecular weight of approximately 31 kD, as determined by SDS-PAGE;
   b) having steroid 5α-reductase activity; and
   c) functioning in the brassinolide biosynthetic pathway.

3. The polypeptide according to claim 1, wherein the amino acid sequence of said protein is substantially the same as the amino acid sequence set forth in SEQ ID NO:2 (Figure 1C).

4. The polypeptide according to claim 1, wherein the amino acid sequence is the same as the amino acid sequence as set forth in SEQ ID NO:2 (Figure 1C).

5. An isolated polynucleotide encoding the DET2 polypeptide of claim 1.

6. An isolated polynucleotide according to claim 5 having a nucleotide sequence as set forth in SEQ ID NO:1 (Figure 1C), or variations thereof which encode the same amino acid sequence, but employ different codons for some of the amino acids, or splice variant nucleotide sequences thereof.

7. A recombinant expression vector containing a polynucleotide sequence according to claim 5.

8. A host cell containing the vector of claim 7.

9. An antibody which binds to the protein of claim 1, or antigenic fragments of said protein.

10. A method of producing a genetically modified plant characterized as having increased yield as compared to a wild-type plant, said method comprising:
contacting a plant cell with at least one nucleic acid sequence encoding a steroid 5α-reductase, said nucleic acid sequence operably associated with a promoter, to obtain a transformed plant cell;
producing a plant from said transformed plant cell; and
selecting a plant exhibiting said increased yield.

11. The method of claim 10, wherein the steroid 5α-reductase is a plant enzyme.

12. The method of claim 11, wherein the plant steroid 5α-reductase is DET2.

13. The method of claim 10, wherein the steroid 5α-reductase is a mammalian enzyme.

14. The method of claim 10, wherein the steroid 5α-reductase is selected from the group consisting of human, rat, mouse and monkey type 1 or type 2 steroid 5α-reductase.

15. The method of claim 10, wherein the contacting is by physical means.

16. The method of claim 10, wherein the contacting is by chemical means.

17. The method of claim 10, wherein the plant cell is selected from the group consisting of protoplasts, gamete producing cells, and cells which regenerate into whole plants.

18. The method of claim 10, wherein the promoter is selected from the group consisting of a constitutive promoter and an inducible promoter.

19. A plant produced by the method of claim 10.

20. Plant tissue derived from a plant produced by the method of claim 10.

21. A seed derived from a plant produced by the method of claim 10.

22. A method for genetically modifying a plant cell such that a plant, produced from said cell produces increased yield as compared with a wild-type plant, said method comprising:
contacting said plant cell with the polynucleotide of claim 5 or a polynucleotide encoding a mammalian steroid 5α-reductase to obtain a transformed plant cell; and
growing the transformed plant cell under plant forming conditions to obtain a plant having increased yield.

23. The method of claim 22, wherein inducing increased growth is achieved by inducing expression of steroid 5α-reductase in the plant.

24. The method of claim 22, wherein the steroid 5α-reductase is a plant enzyme.

25. The method of claim 24, wherein the plant steroid 5α-reductase is DET2.

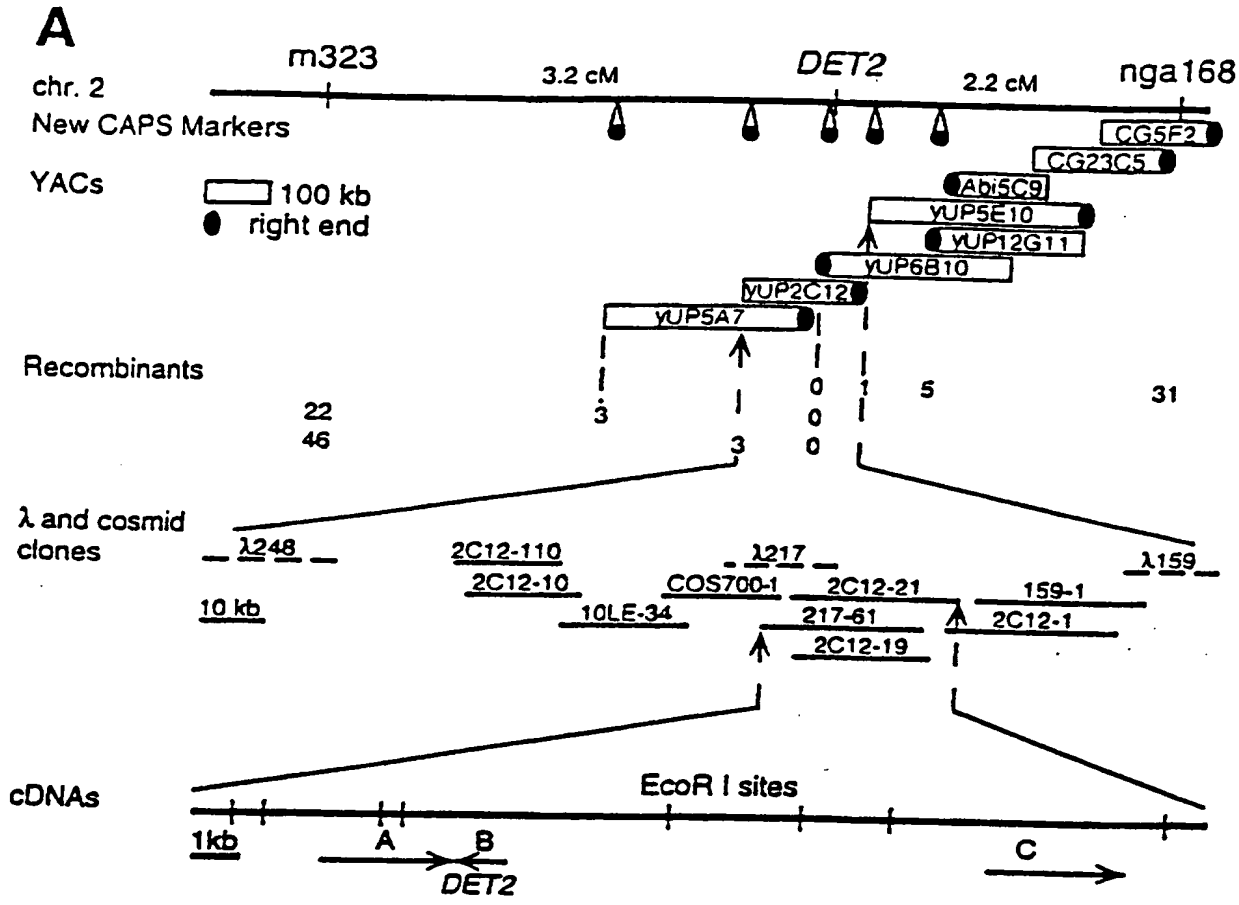
26. The method of claim 22, wherein the steroid 5α-reductase is a mammalian enzyme.

27. The method of claim 26, wherein the steroid 5α-reductase is selected from the group consisting of human, rat, mouse and monkey type 1 or type 2 steroid 5α-reductase.

28. A method of producing a plant characterized as having increased yield, said method comprising:
contacting a susceptible plant with a DET2 promoter-inducing amount of an agent necessary to elevate DET2 gene expression above DET2 expression in a plant not contacted with the agent.

29. The method of claim 28, wherein the agent is a transcription factor.

30. The method of claim 28, wherein the agent is a chemical agent.



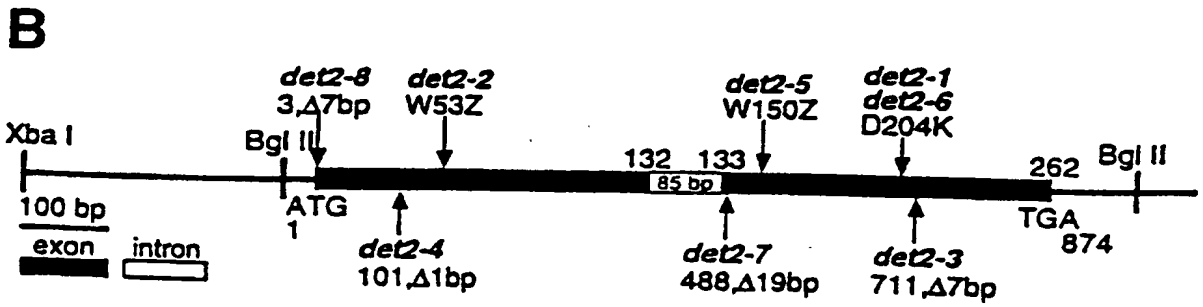


Figure 1A and 1B

FIGURE 1C

DET2 DNA sequence

aattccattaccccgaaaaatggaagaaatcgccgataaaaccttcttccgatactgtctcctcactcttatttttcg
ccggcccaccaaccgccgtccttctgaaattcctccaagctccttacggtaaaacacaaccgtaccggatggggtcc
caccgtatttccaccgattgcttggttcgtcatggagagcccaaccttgtggctcactctcctcctcttcccctttt
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gcactttggtacgattgaagaaagagaaccggggaggttatgtgataccgagaggaggctggttcgagttggtaag
ccgtccgaattatttttggagaggcgattgagtggttgggctgggctgttatgacttggtcttgggccggtattgga
ttttttctgtacacgtgttccaatttgtttccgcgtgcacgtgcgagtcacaagtggtacattgccaagttcaagg
aagagtatttcaagactcgtaaagctgttattccttttgtgtactgagaattgagaaagttgaaaactagtttatc
atatgttatgtgtcaatttgtttccaaactacctttgtcaaaatttccagtaaccggtttaattccaacacggttt
agatcttatgttggtatcttcaacaatgcacaacaaactgtgtattctttagacaaatttta

Deduced Protein sequence:

MEEIADKTFFRYCLLTLIFAGPPTAVLLKFLQAPYGKHNRTGWGPTVSPPIAWFVMESPTLWLTLLLFPFGRHALN
PKSLLLFSPYLIHYFHRTIIYPLRLFRSSFPAGKNGFPITIAALAFTFNLLNGYIQARWVSHYKDDYEDGNWFWWR
FVIGMVVFITGMYINITSDRTLVRLKKENRGGYVIPRGGWFELVSRPNYFGEAIEWLGWAVMTWSWAGIGFFLYTC
SNLFPRARASHKWYIAKFKEEYPKTRKAVIPFVY

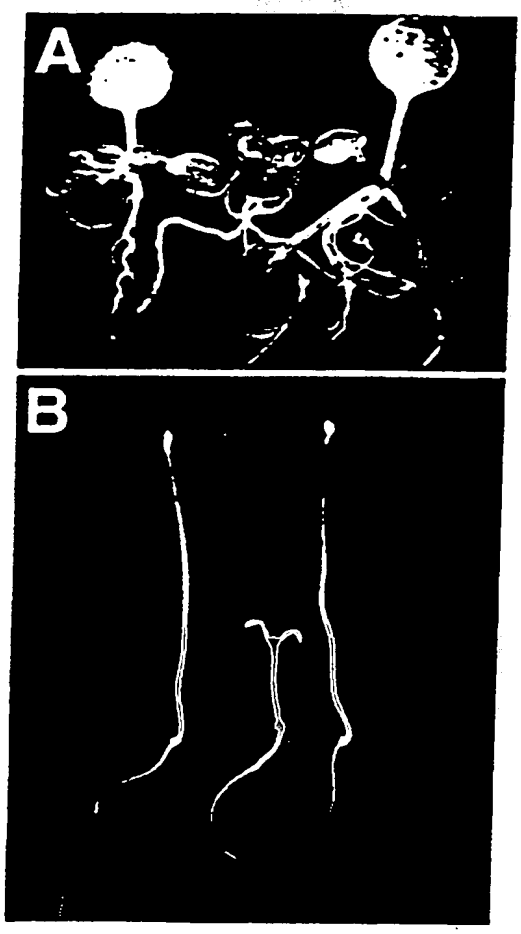

Figure 2

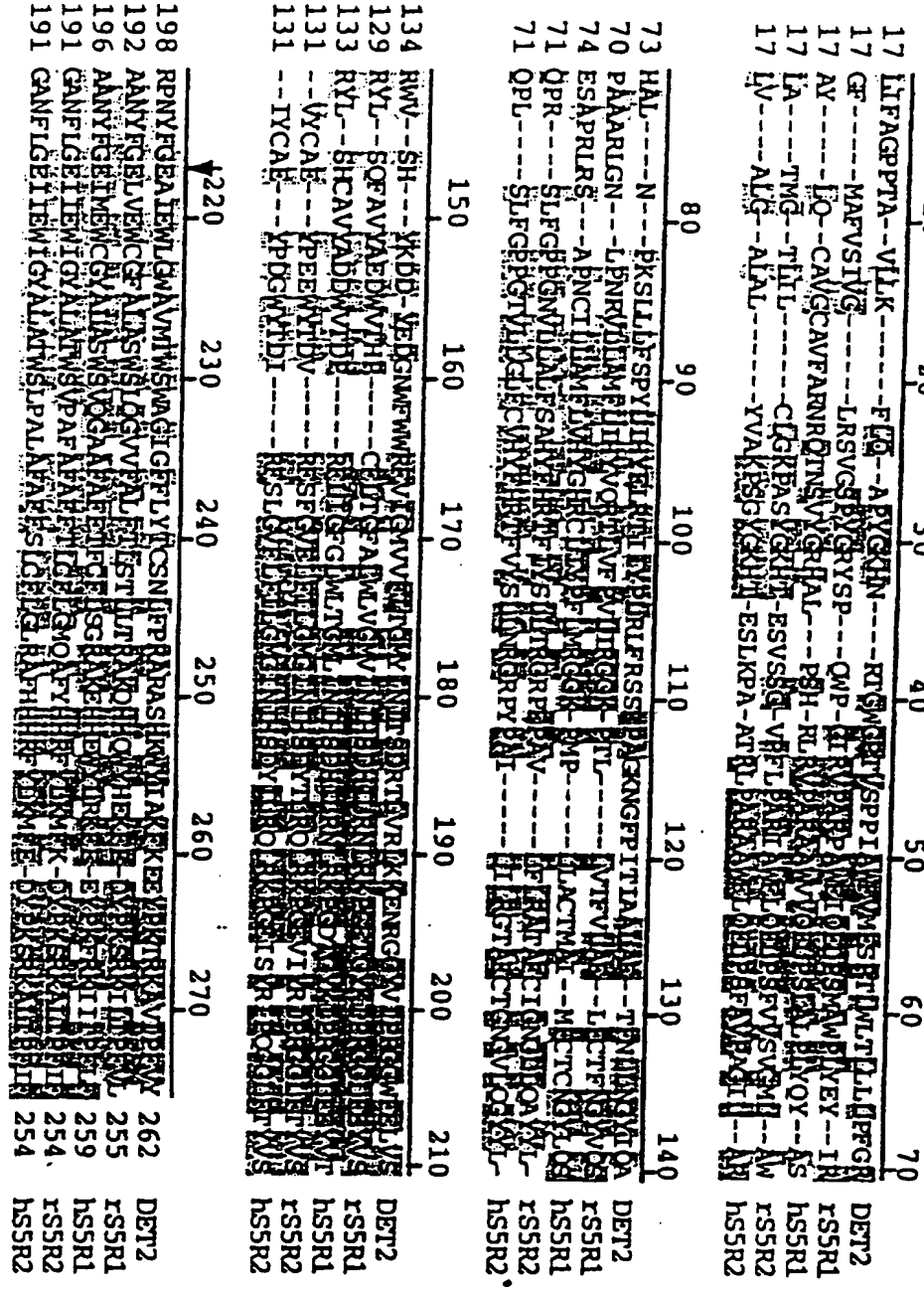

Figure 3A

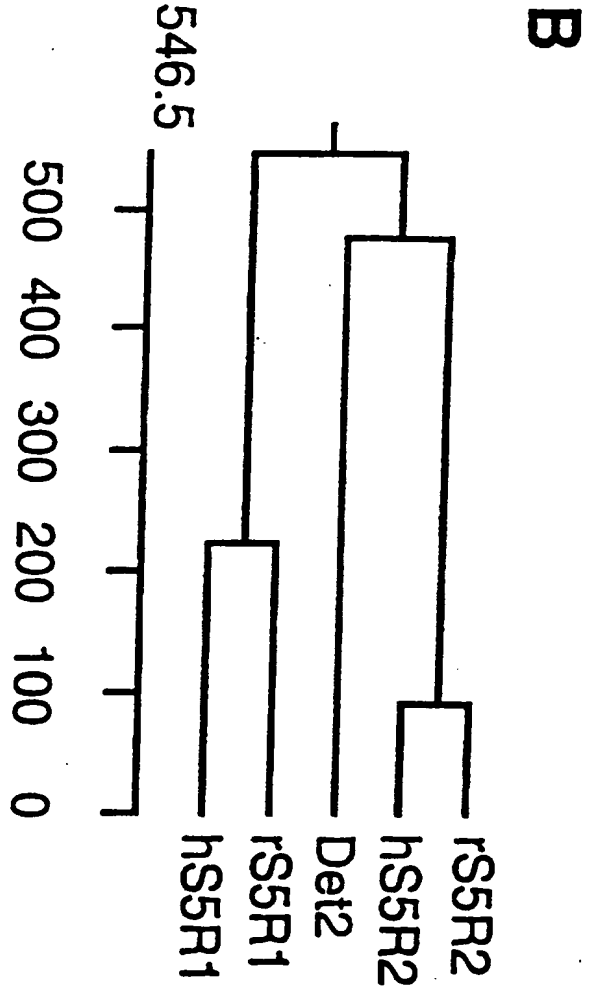

Figure 3B

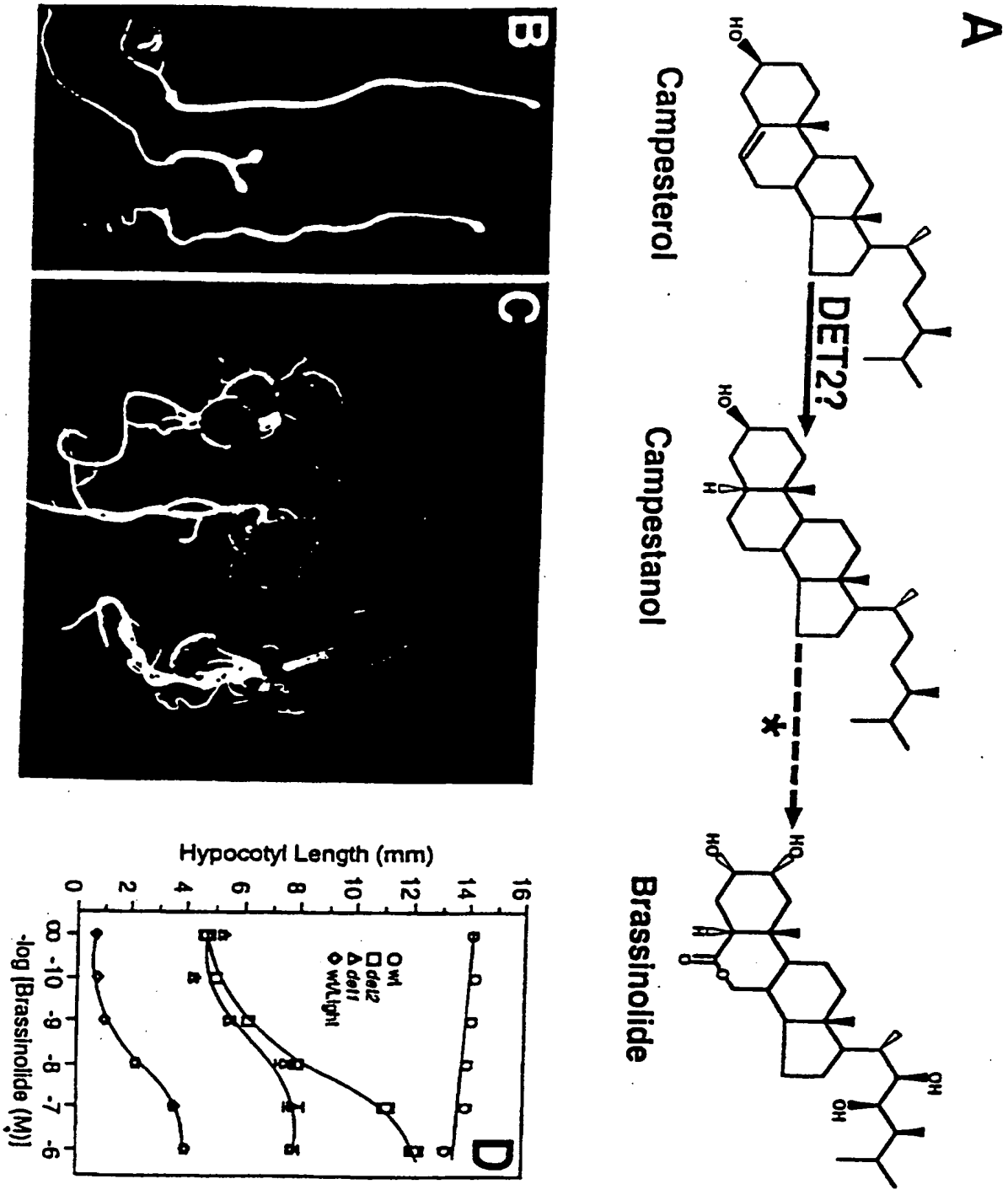
A
Campesterol
DET2?
Campestanol
Brassinolide
B
C
D
Hypocotyl Length (mm)
0
2
4
6
8
10
12
14
16
-log [Brassinolide (M)]
∞
-10
-9
-8
-7
-6
wt
det2
det1
wt light

Figure 4

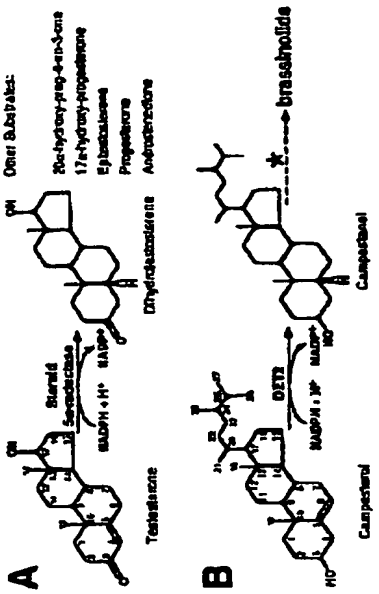

FIGURE 5

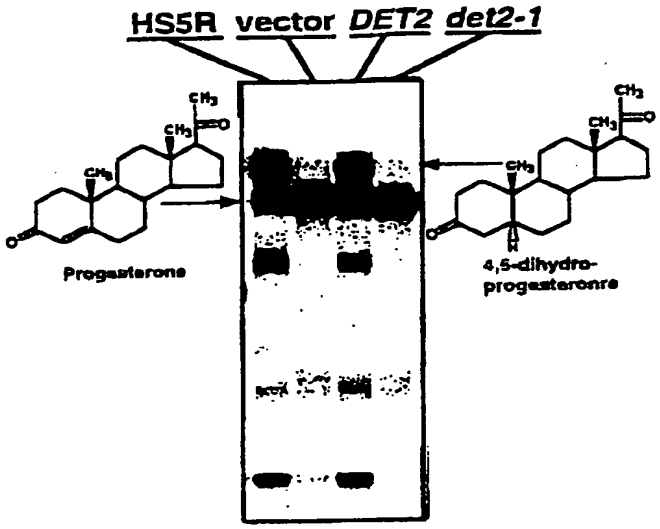

FIGURE 6

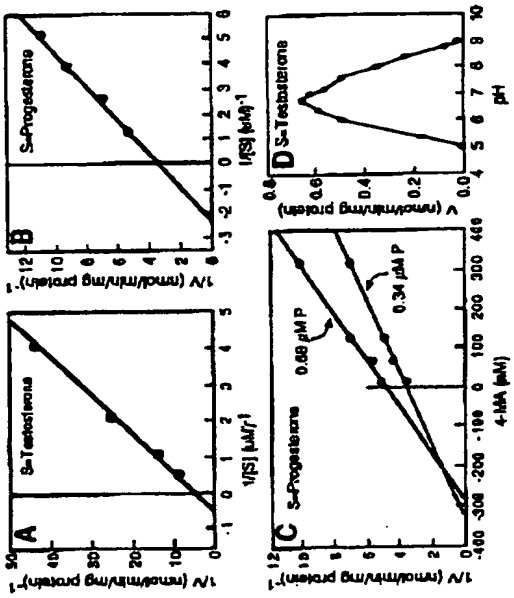

FIGURE 7

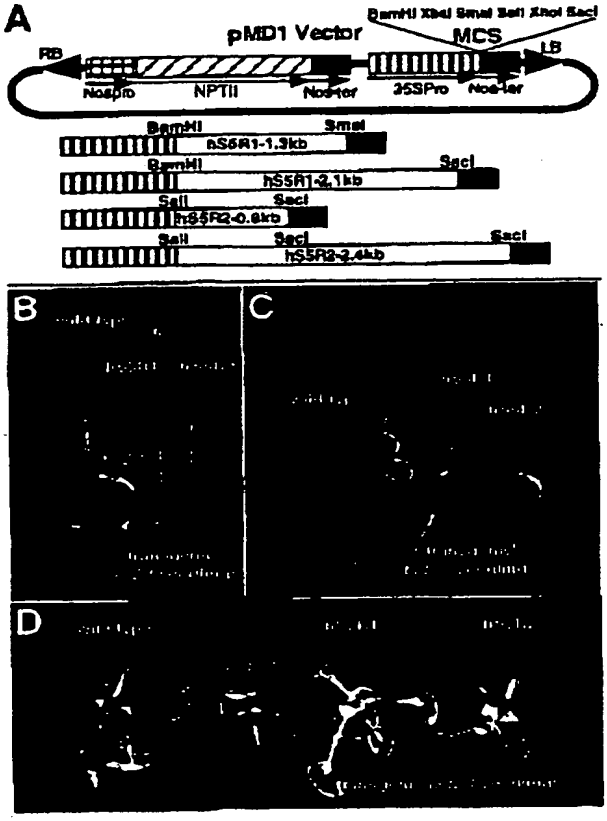
A
pMD1 Vector
MCS
RB
LB
NosPro
NPTII
NosTer
35SPro
NosTer
BamHI
SmaI
hS5R1-1.3kb
BamHI
SacI
hS5R1-2.1kb
SalI
SacI
hS5R2-0.6kb
SalI
SacI
SacI
hS5R2-2.4kb
B
C
D

FIGURE 8

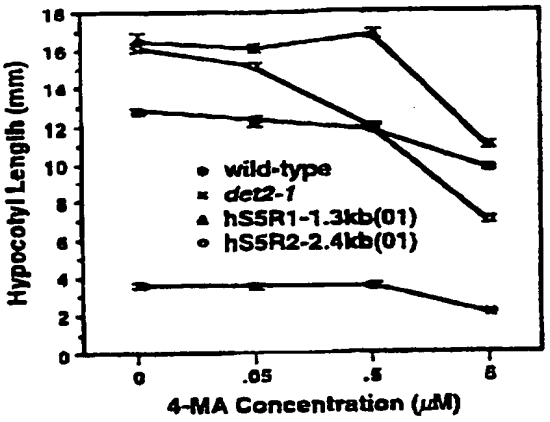
Hypocotyl Length (mm)
18
16
14
12
10
8
6
4
2
0
wild-type
det2-1
hS5R1-1.3kb(01)
hS5R2-2.4kb(01)
0
.05
.5
5
4-MA Concentration (μM)

FIGURE 9

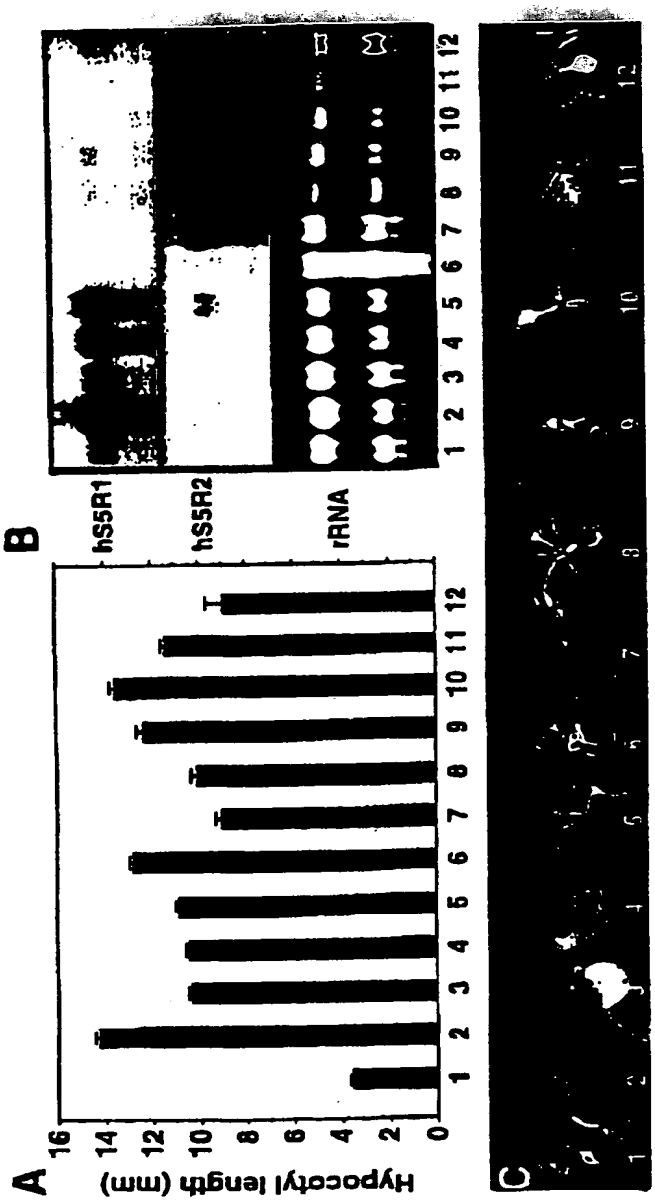
A
Hypocotyl length (mm)
0
2
4
6
8
10
12
14
16
1 2 3 4 5 6 7 8 9 10 11 12
B
hS5R1
hS5R2
rRNA
1 2 3 4 5 6 7 8 9 10 11 12
C

FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06115

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205; 435/69.1, 172.3, 320.1, 419; 536/23.5, 23.6, 24.1; 436/71, 139, 817

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CABA, CAPLUS, MEDLINE, BIOSIS
search terms: DET2, alpha reductase, brassinosteroid, plant, transgenic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
A, P	RUSSEL D. W. Green light for steroid hormones. Science. 19 April 1996, Vol. 272, No. 5260, pages 370-371.	1-30
T	LI et al. A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science. 19 April 1996, Vol. 272, No. 5260, pages 398-401.	1-30
A, P	SZEKERES et al. Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell. 19 April 1996, Vol. 85, No. 2, pages 171-182.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
01 AUGUST 1997

Date of mailing of the international search report
13 AUG 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer
THOMAS HAAS
Telephone N . (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International applicatic No.
PCT/US97/06115

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 9/00, 9/02, 15/00, 15/09, 15/12, 15/29, 15/82; A01H 4/00, 5/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

800/205; 435/69.1, 172.3, 320.1, 419; 536/23.5; 23.6, 24.1; 436/71, 139, 817